MICROBIAL PROGRAMMING OF THE NEONATAL PIG

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GENERAL ABSTRACT

Microbial succession, composition and ecological distribution within the gastro-intestinal tract are critical areas of study since commensal bacteria have been shown to affect animal health and development. A series of experiments were conducted to determine whether altered microbial succession in neonatal animals would modulate the development and health of pigs later in life. An initial experiment in conventional pigs was conducted to establish the early postnatal microbial succession profile and to identify early colonizing bacterial species. Culture-independent analysis of digesta and mucosal microbiota showed distinct variation between the proximal and distal gastro-intestinal tract (GIT) indicating that fecal or distal gut profiles cannot be used to predict succession in the upper GIT. Temporally, *Clostridium* spp. were found to be most prevalent in the GIT microbiota of the neonatal pig up to 0.5 d of age, accompanied by a high abundance of *Escherichia* and *Shigella* spp. These genera were transiently displaced by *Streptococcus* spp. followed by a preponderance of *Lactobacillus* spp. between 3 and 20 d of age. Subsequently, a “snatch-farrow” model was employed to modulate early postnatal microbial succession and investigate the effects on postweaning microbial composition. Pigs were collected into sterile towels directly from the vaginal canal and transferred to a sterile isolator environment for the first 4 days. Pigs were either inoculated with sow feces or not at 1 d of age resulting in significant differences in fecal microbial profile at 4 days of age, prior to removal from isolators. Analysis using terminal restriction fragment length polymorphisms (TRFLP) of intestinal microbiota at 28 d of age did not show significant clustering or variation in diversity indices for either group during the 4-d postnatal isolator phase. However, enumeration of selected taxa using quantitative PCR did indicate significant treatment differences in postweaning microbiota. Despite these results, this approach was rejected for further use as the

protocol provided only moderate control of early postnatal colonization and variation and unpredictability of the timing of natural farrowing contributed to significant litter effects. Finally, a gnotobiotic monoassociation model was used investigate the effects of modulating early postnatal microbial succession on postweaning physiology, microbial composition and mucosal gene expression. Twenty-four cesarean-section derived piglets were monoassociated for the first 4 days of life with either *L. mucosae* (L), *S. infantarius* (S), *C. perfringens* (C) or *E. coli* (E). Pigs from treatments E and L animals showed the highest growth rate during the conventional rearing period (7-28 d of age). Monoassociation with different bacterial species during the first 4 d of life resulted in significant changes in postweaning microbial composition in small intestine and colon as assessed by quantitative PCR, although TRFLP did not identify unique clustering by treatment or variation in diversity. *L. mucosae* was the only inoculant species with significant variation, with a reduction in the colonic mucosa at 28 days of age. Monoassociation with *L. mucosae* was also associated with increased nutrition related gene expression in small intestine. Pigs monoassociated with *E. coli* had low expression of microbial sensing (*TLR2* and *4*), *NFκB* complex genes and mucins at 28 d of age. This study clearly showed that controlled early microbial succession in neonatal pigs altered post-weaning commensal microbiota composition, postweaning physiology and host gene expression in small and large intestine. The findings suggest the importance of peri-natal management and feeding strategies in promoting postweaning health and performance.

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DEDICATION

To Alfred and Claudia Petri,

without whom, I would never have come this far.

Ohne Euch hätte ich das nie geschafft.

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LIST OF ABBREVIATIONS

AB2.5/PAS	1% alcian blue pH 2.5 with periodic acid and Schiff base reaction
ACTB	beta-actin
ADG	average daily gain
AEEC	Attaching and effacing *E. coli*
AIDA	adhesin involved in diffuse adherence
APC	antigen presenting cells
APN	Alanyl aminopeptidase
Banim	*B. animalis*
BaPr	B*acteroides*/ *Prevotella* spp.
Bifido	*Bifidobacterium* spp.
BLAST	basic local alignment search tool
BLUP	linear unbiased prediction
bp	base pairs
Btham	*B. thermacidophilum*
BW	body weight
Ccl1	*Clostridium* cluster I
Ccl14a	*Clostridium* cluster XIVa
Ccl4	*Clostridium* cluster IV
CDAD	*C. difficile* associated disease
cDNA	complementary deoxyribonucleic acid
co	colon
CpA, CpC	*C. perfringens* type A, C
Cperf	*C. perfringens*
CpG	Cysteine-phosphate-Guanine
*cpn*60 UT	*chaperonin*60 universal target
Ct	threshold cycle
DC	dendritic cell
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DNA	deoxyribonucleic acid
DNMT	deoxyribonucleic acid (cytosine-5)-methyltransferase
dNTP	Deoxyribonucleotide triphosphate
Eae	attaching and effacing factor
Ecocc	*Enterococcus* spp.
EDEC	edema disease *E. coli*
EGF	epidermal growth factor

ELISA	enzyme-linked immunosorbent assay
Entero	Enterobacteria
ETEC	Enterotoxin producing *E. coli*
FISH	fluorescent *in situ* hybridization
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIT	gastro-intestine tract
GLP-2	glucagon-like peptide-2
GLP-2R	glucagon-like peptide-2 receptor
H&E	hematoxylin and eosin
HAT	histone acetyltransferase
HDAC	histone deacetylase enzyme complex
HDAC1	histone deacetylase enzyme complex 1
HEPA	high-efficiency particulate arresting
HSD	honest significant difference
IEC	intestinal epithelial cell
IFN-γ	interferon-γ
IgA, IgG, IgM	immunoglobulin A, G and M
IGF	insulin-like growth factor
IL	interleukin
iNKT	invariant natural killer T
IU	international unit
IW	initial weight
Jej	jejunum
Lacto	*Lactobacillus* spp.
Lamyl	*L. amylovorus / L. sobrius*
LB	Luria Bertani
Ldelb	*L. delbrueckii*
LI	large intestine
Ljohn	*L. johnsonii*
LPH	Lactase-phlorizin hydrolase
Lreut	*L. reuteri*
LT	heat labile enterotoxin
MBP	5-methyl-cytosine binding protein
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NFKBIA, IκBα	NFκB inhibitor α
O.D.	optical density
Ori.	orientation
PAGE	poly-acrylamide gel electrophoresis

Pbucc	*P. buccalis*
PCR	polymerase chain reaction
PepT1	Peptide transporter 1
PGF	prostaglandin F2α
PWD	post weaning diarrhea
qPCR	quantitative polymerase chain reaction
RDP	Ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
SEM	standard error of the mean
Sequi	*S. equinus*
SGLT	sodium-glucose transporter
SI	small intestine
Sinfa	*S. infantarius*
Ssuis	*S. suis*
STa, STb	heat stabile enterotoxin
sto	stomach
Strepto	*Streptococcus* spp.
Stx2e	Shiga-like toxin
TcdA, TcdB	monomeric enterotoxin A, B
Temp.	annealing temperature
TGE	transmissible gastroenteritis
TGF	transforming growth factor
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TotBac	total bacteria
TRF	terminal-restriction fragment
TRFLP	terminal-restriction fragment length polymorphism
VFA	volatile fatty acid
Vh:Cd	villus height to crypt depth
vs.	versus
ZO-1	zona occludens 1

1.0 GENERAL INTRODUCTION

Concern that antibiotic use in livestock production contributes to antibiotic resistance towards pathogens for humans, has increased the regulation of prophylactic and therapeutic use of antibiotics for livestock animals and led to a total ban on use of antibiotics as growth promotants in the European Union since 2006. The need for efficacious alternatives to prophylactic antibiotics, has driven research aimed at identifying alternative strategies to modify intestinal microbial colonization patterns (Kelly 1998) as a mechanism to maintain health.

The gastrointestinal tract is one of the most dynamic and complex examples of a microbial ecosystem. Initial colonization of the intestinal tract proceeds rapidly after birth by bacteria both of maternal and environmental origin. Despite the potential diversity of bacteria in the environment, the sequence of microbial succession appears to be predetermined and remarkably similar across species (Conway 1996). The swine microbiota, which is estimated to contain several hundred different bacterial species, eventually attains a "stable" composition and it is this stability that has, for decades, been considered to confer significant health benefits to the host, and to play an important role in mucosal defense (Kelly 1998; Turnbaugh *et al.* 2006). Although the adult intestinal microbiota is considered stable, it can be perturbed by alterations such as stress, antibiotic administration or dietary shifts such as those that occur for sows at weaning (Kelly *et al.* 1998; Thompson *et al.* 2008; Mulder *et al.* 2009).

More recently, the role of the intestinal bacteria has been recognized in development and regulation of the host immune response as well in the etiology of non-transmissible diseases such as inflammatory bowel diseases, cancer and obesity (Turnbaugh *et al.* 2006; Guo *et al.* 2008; Thompson *et al.* 2008). In spite of this research effort, there is still a lack of understanding in the

mechanisms governing bacteria/host cell interactions, and the implications for the host when microbial colonization patterns are altered. The microbial composition has important implications for the host; for example, *B. thetaiotaomicron* colonization leads to intestinal maturation in germ free mice (Hooper *et al.* 2001). In addition to specific microbiota-host interactions, long-term effects due to changes in microbiota composition early in life are believed to have long lasting effects on host immune function (Kelly et al. 2007). Both aspects of beneficial microbiota and their long-term effects are leading to the idea of microbial programming.

The lack of knowledge on microbial colonization is due in part to the difficulties of identifying key bacteria responsible and physiological mechanisms in the highly diverse gastrointestinal ecosystem of a conventional animal (Savage 1977). In an attempt to define the relationship of individual organisms with the host, previous research has used *in vitro* culture of cell lines combined with bacteria; however, these systems usually contain a single cell type and lack the inherent physiological complexity of an *in vivo* system (Willing 2007). The use of a gnotobiotic animal model overcomes both the challenge of the microbial diversity while simultaneously allowing the study of physiological and developmental processes that occur with interactions along the host microbial interface. Development of the gnotobiotic pig model has helped to characterize the response of the pig to different bacterial species and will continue to serve as a key tool for linking variation in host response to specific members of the complex intestinal microbial community. Identifying specific mechanistic links betweeen bacterial community composition and gastrointestinal function is a critical component in developing nutritional and management strategies for improved swine production

Based on previous research that shows there are changes in microbiota in the digestive tract of the neonatal pig up to and post weaning, differences in impact of the varying major bacterial species on pig gut development are of foremost interest. Furthermore, it is necessary to determine the optimal neonatal microbial environment in the digestive tract of the newborn pig and to improve our understanding microbial succession of neonatal animals in order to develop tools to modify and control commensal bacteria thereby maintaining animal health and productivity without antibiotic growth promoters. A review of microbial ecology and succession, pig gastro-intestinal tract development, maternal influences on neonatal development, microbial influences on pig gastro-intestinal development and evidence for long term effects of early postnatal environment follow as background to establishing an experimental hypothesis and thesis objectives.

2.0 LITERATURE REVIEW

2.1. Microbial Ecology and Succession in Mammals

2.1.1. Digestive Microbial Ecology

The gut of the mammal is an open and integrated ecosystem (Gordon and Pesti 1971), consisting of six major areas being oral cavity, esophagus, stomach, small intestine (SI), cecum and colon. The structure and localized secretions of the gastrointestinal tract dictates the location of the microbiota and, to some extent, the composition of the microbiota as well. Rene Dubos and his group were the first microbiologists in the early 20th century to systematically explore the gastrointestinal microbial ecology. They maintained murine colonies under various conditions and investigated microbial changes finding remarkable microbial composition changes within the gut over time and under different rearing conditions (Dubos *et al.* 1966).

Microbial habitats in the gastrointestinal tract may exist in any area from the oral cavity to the anus (Savage 1977), in the lumen, on an epithelial surface, or deep in the crypts of Lieberkühn (Savage 1975; Mackie *et al.* 1999). The lumen can be colonized by microbes in any area of the tract, with an approximate count of 10^3 to 10^5 CFU/g of content in the stomach and proximal SI with increasing counts towards the distal SI with 10^8 CFU/g of content. Major colonization occurs in the hindgut with 10^{10} to 10^{11} CFU / g of content promoted by the slower content flow rate which does not exceed the doubling rate of the microbial population (Savage 1977; Mackie *et al.* 1999). The epithelial surface also can be colonized in any area by characteristic biotas and similarly, in some animal species, the mucosal crypts may possess bacteria distinct from either the luminal or epithelial biotas.

The overall commensal gut microbiota of the healthy mammal is in an ever changing equilibrium with both a native and a transient component. Some bacteria colonize and become temporarily or permanently indigenous, whereas others are non-indigenous and enter from the feed/environment imparting a short-term impact on the digestive environment. Once specific niches are colonized by indigenous microbiota, these microbes shed from their now native habitat to migrate through other habitats within the gastro-intestinal canal without colonizing (Savage 1977).

2.1.2. Microbial Succession Patterns

During birth, the sterile GIT (Gordon and Pesti 1971) is initially exposed to the microbiota from the maternal birth canal and then subsequently introduced to various other environmental sources of microbes (Gracey 1982; Chen *et al.* 2005). In general, the gut is first colonized by aerobic and facultative anaerobic bacteria, which reduce the oxygen content in the GIT creating an oxygen-limited environment and allowing facultative and obligate anaerobic bacteria to colonize (Smith 1961; Swords *et al.* 1993; Mackie *et al.* 1999; Buddington 2003). With the initial introduction of bacteria into sterile habitats, a sequence of events begins that is unique to the animal genotype, and a number of environmental factors (Savage 1977). Successive colonization of the various GIT habitats by initial microbes occurs until all habitats are occupied by indigenous communities (Alexander 1971).

Populations of the GIT are initially dominated by facultative anaerobes but then shift towards obligate anaerobes within the first days of life (Mackie *et al.* 1999). Typical representatives of early neonatal colonization are facultative anaerobes such as Enterobacteria and *Streptococcus* spp. as well as *Lactobacillus* spp. (Savage 1977; Swords *et al.* 1993; Mackie *et al.*999). In some animal species, these bacterial types may gain temporary predominance (Smith 1961; Schaedler *et al.* 1965; Smith 1965; Savage *et al.* 1968) and may be found at these high levels in all regions

of the tract including the stomach (Savage *et al.* 1968; Tannock and Smith 1970). The stomach and duodenum are suggested to have a relatively low microbial density and diversity due to the acidic environment, bile secretions such that the microbial community is dominated by the *Lactobacillus*, *Streptococcus* and *Enterococcus* genera. The ability to attach to and colonize the mucosal layer is important in the succession of bacterial species in the proximal SI as rate of passage is rapid (Laux *et al.* 2005). As the rate of digesta passage slows towards the distal SI, phylogenetic diversity increases to include not only those genera predominant in the stomach and duodenum, but also genera within the Clostridiales and Bacteroidetes taxonomic families (Hill *et al.* 2004; Richards *et al.* 2005). In these regions, the mucus layer serves as a matrix and a source of nutrients supporting bacterial replication (Laux *et al*. 2005). The complexity of the microbial population is highest in the hindgut and is generally dominated by the low G+C Gram-positive groups including *Clostridium*, *Bacillus*, *Ruminococcus* and *Fusobacterium* (Hill *et al.* 2002; Leser *et al.* 2002).

2.1.3. Microbial Succession Patterns in the Pig

Microbial colonization succession in the pig has received limited attention, and in large part, that attention has been focused on the feces or distal colon due to ease of accessibility for sampling. Like other mammals, early GIT colonization in pigs begins at birth with an introduction to the population of bacteria in the vaginal tract and subsequently sows feces (Tannock *et al.* 1990). Bacteria quickly inhabit the entire pig GIT including the gastric epithelium (Savage *et al.* 1968; Tannock and Smith 1970) and within the first 12 hours of birth, the distal colon of the pig is already colonized with 10^9 to 10^{10} bacteria per gram of digesta (Swords *et al.* 1993). Janczyk *et al.* (2007) found that in the pig feces, *Lactobacillus* spp. peak in abundance at approximately 2 days of age and that by 5 days of age *Clostridium* spp. are predominant (Janczyk *et al.* 2007). Changes in the abundance and diversity of both the

Lactobacillus and *Clostridium* spp. have been shown to fluctuate throughout time, particularly at weaning, where after *Bacteroides* spp. become more prevalent in the adult colonic microbiota of the pig (Swords *et al.* 1993). Whether microbial succession patterns in proximal small intestine mirror those observed in colon and feces is not yet clear; however, molecular based profiling studies in the pig have indeed demonstrated marked differences in microbial composition between proximal and distal intestinal locations (Konstantinov *et al.* 2004; Hill *et al.* 2005; Richards *et al.* 2005). Research examining post-natal development of the porcine microbiota in ileal digesta collected at 2 days of age, weaning, and post-weaning has identified *E. coli*, *S. flexneri* and *L. sobrius*, *L. reuteri* and *L. acidophilus* as the early dominant species in the ileum (Konstantinov *et al.* 2006). The process of succession continues on past the early stages of life and generally a significant change occurs once mammals begin to sample solid food. At this time, strict anaerobes are generally detected as being predominant in the large intestine. Population levels of these anaerobic bacteria increase progressively and by weaning, populations are considered to be at adult core microbial community concentrations and completely dominate all other microbial populations in the large bowel (Savage *et al.* 1968; Tannock and Smith 1970). As the quantities of strict anaerobe bacteria increase, the level of facultative anaerobe bacteria, such as Enterobacteria and *Streptococcus* spp. may decline concomitantly (Smith 1961; Schaedler *et al.* 1965; Smith 1965; Savage *et al.* 1968).

Already before the piglet is exposed to the barn environment, insufficient oxygen supply to the neonate during birth can result in intestinal dysfunction and increased incidences of enterocolitis later in life however, the direct mechanism is unknown (Cohen *et al.* 1991; Powell *et al.* 1999). The immediate environment after birth is of utmost importance, and has proven to have direct impact on gut development of the neonate. Essential extrinsic factors include the

bacterial load of the environment, which for pigs mostly depends on the composition of the maternal gut microbiota, microbiota from the birth canal, diet, the mode of delivery and medication (Fanaro *et al.* 2003). Microbes from the vaginal canal and the perineal area enter the mouth and the stomach of vaginally delivered infants and, within few minutes after birth, the gastric content of the newborn is influenced by and reflects the cervical flora of the mother of humans (Mackie 1999). When infants are born by caesarean section and therefore not exposed to maternal vaginal and fecal flora, the surrounding environment is extremely important for the intestinal colonization. Furthermore, these babies may be separated from their mothers for long periods after birth (Adlerberth 1999). In infants born by caesarean delivery, the establishment of a stable flora, which is characterized by a low incidence of *Bacteroides* spp. and a low isolation of other bacteria, is consistently delayed (Fanaro *et al.* 2003; Palmer *et al.* 2007). Delivery by caesarean section combined with maternal antibiotic prophylaxis prior to delivery in humans showed a prolonged delay of Bacteroides colonization that was still evident at 6 months of age (Grölund *et al.* 1999). In developing countries, a pronounced bacterial exposure from the environment may induce an unstable colonization pattern and favor the presence of pathogens (Adlerberth 1999). In these societies, exclusive breastfeeding is important to develop a microbiota with a low pathogenic potential. Comparable research has not been performed in pigs, and even though it is likely that colostrum feedings reduce pathogenic potential, it is unclear if the results are directly transferable. It was determined that pigs are growing faster, more efficiently and are less variable in size due to breeding efforts, however gut morphology has not yet made it into the list of breeding goals, except for *E. coli* K88 or F4 and F18 fimbriae receptor suppression (Thomson 2006). Therefore, health development and growth performance

of the new born piglet in a standard production environment is highly dependent on management factors like herd health management/status and the hygiene management of the production site.

2.1.4. Factors Affecting Succession

Phenotypic plasticity is the change in the expression phenotype of a genotype based on the influence of environment (Scheiner, 1993). Successional events involve complex sequential interactions between the animal genotype, diet, GIT micro-environments, and the diverse microbial types. These interactions influence the localization and the succession of the developing microbial communities as well as dictate their final composition resulting in a wide variety of phenotypes (Savage 1977; Van der Wielen *et al.* 2000; Apajalahti *et al.* 2001; Hill *et al.* 2005).

2.1.4.1. Genotype Effects

Although host factors are known to influence microbial composition, the influence of any particular factor on the composition of the biota in any habitat is not so clear. Studies looking at a variety of species of mammals have yielded inconsistent results regarding the impact of genetics on microbial succession (Spor *et al.* 2011). However, research looking at digestive microbiota in monozygotic and dizygotic twin pairs in humans showed that related individuals have more similar intestinal microbial profiles than unrelated individuals, indicating an influence of host genotype. While similarities between the microbiota of related individuals could be due to a shared environment, the influence of host has also been shown in other species (Weimer *et al.* 2010). Research in cattle has shown that in a mature rumen microbial ecosystem, a host animal can reestablish its characteristic ruminal pH, VFA and bacterial community composition with relative success despite a dramatic perturbation of its microbial community by the addition of ruminal contents from different animals (Weimer *et al.* 2010). Furthermore, using a candidate-

gene approach, where one gene is deleted or added to a model host, it has been shown that a single gene can tremendously impact the diversity and population structure of the gut microbiota (Spor *et al.* 2011). This has been shown in pigs where polymorphisms of the *MUC20* gene resulted in the mucin adhesion and proliferation of *Escherichia coli* F4/K88, a known major cause of major cause of diarrhea and death in neonatal and young pigs. Therefore, susceptibility to ETEC F4 is inherited as a monogenic trait in pigs (Ji *et al.* 2011) and the host genotype, impacting the immunological response can trigger a change in microbial community structure by giving advantage to some microbial species (Thompson et al. 2008).

2.1.4.2. Diet Effects

To measure the impact of host genetics on microbial diversity, it is necessary to understand the additional factors which can influence variation in the microbiota in the absence of host genetic variation. Especially as often times these environmental factors can mask host genetic effects (Spor *et al.* 2011). Consistently, diet has been shown to play a significant role in GIT microbial succession. A clear example of the impact of diet can be seen in human infants. Breastfed infants are found to have predominantly *Bifidobacterium* spp. in their feces compared to a more diverse fecal microbiota, with a greater abundance of *Bacteroides* spp., in formula-fed infants (Harmsen *et al.* 2000; Apajalahti *et al.* 2001). The likely explanation for this is that components of human milk contain non-absorbable oligosaccharides, nucleotides and gangliosides, which affect colonization by *Bifidobacterium* (Kunz and Rudloff 1993; Wharton *et al.* 1994; Rueda *et al.* 1998). However, in spite of the potential impact of diet, diversity of intestinal microbial populations between adult humans is surprisingly high even with similar diets and lifestyles (Moore *et al.* 1978). As previously stated, particularly profound transitions in the succession take place when the mammal begins to consume solid food (Swords *et al.* 1993; Deplancke *et al.* 2000; Buddington 2003). This can be even more of a profound impact on the

microbial composition of the GIT in systems where the process of weaning and the transfer to solid food is abrupt, as it is in many managed food animals such as the pig (Lecce *et al.* 1979).

2.1.4.3. Impacts of Genotype and Environment

Some of the more current research clearly shows the impact of both the host genotype and the environment combined. The "cohabitation effect" as shown by Thompson *et al.* (2008) describes the onset of a transition where, despite previous individual variation, co-housed piglets suddenly develop very similar communities around 21 days of age. The onset of this change after 2 weeks is predicted to be after the development of key elements of the host immune system and before significant levels of IgA were observable (4 weeks). The outcome of this research was that there was dramatic alteration of the GIT microbial community resulting in significant similarity between the stable GIT communities of cohabiting pigs. Furthermore, this change was then seen to persist up to 420 d of age (Thompson *et al.* 2008) implying that the impact of cohabitation resulted in a long term effect on microbial succession.

One of the most dramatic impacts of environment on the succession patterns can be seen in the comparison premature infants to healthy full term infants. Microbial acquisition and succession are altered in premature infants due to a number of factors including limited maternal contact, delayed initiation of enteral feedings, exposure to antibiotics, and infection control procedures which minimize exposure to environmental organisms (Hoyos 1999). Under these conditions, it is believed that pathogenic bacteria are more likely to colonize due to the lack of or the competitive inhibition of a normal commensal bacteria and/or the slower maturation in mucosal barrier mechanisms (Hoyos 1999). Common in premature infants, associated with introduction of enteral feedings, is the colonization and overgrowth of with pathogenic bacteria including Enterobacteriaceae, *Clostridium* and *Staphylococcu*s species, and development of often

fatal necrotizing enteritis (NEC). All of these factors constitute a variety of environmental effects that come together to create a dramatic influence on GIT microbial succession.

2.1.5. Neonatal Pig Pathogens

2.1.5.1. Coccidiosis

Infectious diarrhea is a very common issue in nursery piglets. Diarrhea in the preweaned pig, if not caused by malnutrition, is mostly caused by the presence of pathogenic viruses, bacteria or parasites. Neonatal coccidiosis is caused by the protozoa *Isospora suis* (Lindsay *et al.* 1999), which is responsible for approximately one fifth of all cases of piglet diarrhea in the United States (Stuart and Lindsay 1985). The parasite forms sporulated oocytes or sporocytes which are nearly impossible to eliminate from the environment and require specific procedures and cleaning agents (Martineau *et al.* 1995). The sporocytes are activated by digestive enzymes and bile salts. The now infectious sporozoites of *Isospora suis* penetrate the enterocyte of the jejunum and ileum, the caused cell damage leading to diarrhea (Lindsay *et al.* 1980). Anticoccidial drugs are routinely used for prophylactic disease control in peri-natal pig production (Martineau *et al.* 1995).

2.1.5.2. Rotavirus and Coronavirus

Viral infections in neonatal pigs are commonly caused by rota- and coronaviruses. Rotaviruses are species specific (Hoshino and Kapikian 1994); rotavirus type A being the most commonly represented in neonatal pigs (Paul *et al.* 1990). Serogroup typing is based on structural differences of the VP6 protein of the virus (Estes and Cohen 1989); serogroups A-E have been diagnosed in pigs (Paul and Stevenson 1999). Rotaviral ribonucleic acid (RNA) is double stranded (Hoshino and Kapikian 1994) whereas the transmissible gastroenteritis virus, a coronavirus, contains single stranded RNA (Saif and Wesley 1999). Rotaviral enteritis and Transmissible Gastroenteritis (TGE) can both occur right after birth until post weaning.

Necropsy examination of affected pigs reveal thin small intestinal walls, villous atrophy and empty lacteals. Enzyme-linked immunosorbent assay (ELISA), reverse-transcription polymerase chain reaction (RT-PCR), poly-acrylamide gel electrophoresis (PAGE) and/or fluorescent antibody testing are needed to confirm infection (Thomson 2006). Rotavirus vaccines are available (Taylor 1995); however, TGE can only be treated or eradicated by a strict quarantine and feed-back regime if implemented right after the herd is confirmed positive (Harris 2000). The enzootic form, leading to suboptimal performance in nursery and grower production, is caused by uninterrupted oral-fecal transmission of viruses by carrier animals (Martineau *et al.* 1995). Viral or parasitic infections often lead to concurrent infections with pathogenic bacteria, for example with *E. coli* (Taylor 1995).

2.1.5.2. *Escherichia coli*

Non-virulent and virulent *E. coli* are part of the commensal microbiota, the non-virulent *E. coli* outnumbering the virulent ones at least 5:1 in the healthy pig (Fairbrother and Gyles 2006). Since *E. coli* virulence mechanisms differ by strain, pathogenic *E. coli* is categorized by pathotype (Gyles and Fairbrother 2004). Enterotoxin producing *E. coli* (ETEC) causes colibacillosis between 0 and 6 d of age and is a pathogen contributing to post weaning diarrhea (PWD) syndrome 1 to 3 weeks after weaning (Saif and Wesley 1999). They produce one or more enterotoxins, being either heat labile (LT) enterotoxin or heat stabile enterotoxin STa/STI, STb/STII and EAST1, and may occasionally also produce Shiga-like toxin (Stx2e). They adhere to the pig's intestinal epithelial cells (IEC) by one or more fimbrial adhesins F4 (also K88), F5 (K99), F6 (987P), F18 (post weaning only), F41 or by means of an adhesin involved in diffuse adherence (AIDA). Edema disease *E. coli* (EDEC) mainly produces the heat stabile enterotoxin STx2e, also called edema disease principle or vasotoxin, while carrying the same adhesive factors as ETEC in PWD (Fairbrother and Gyles 2006). Attaching and effacing *E. coli* (AEEC)

are similar to enteropathogenic *E. coli* (EPEC) in humans (Helie *et al.* 1991). They attach to the pig's IEC via EPEC attaching and effacing factor (Eae; also called intimin), wearing off villous cell surfaces and invading its epithelial cells. In general, none of the classic *E. coli* virulence factors are present in AEEC, but mixed PWD or EDEC infections with AEEC are common (Zhu *et al.* 1994). Secretory diarrhea caused by ETEC and EDEC is watery yellowish with a range of pH 7-8. Villi atrophy is not observed during autopsy, lacteals are filled and the mucosa shows edema and is congested. Gram negative rods can be observed by inspection of small intestinal epithelium by microscopy. Supportive treatment of disease and/or antibiotic administration are common, vaccines are available (Fairbrother and Gyles 2006).

2.1.5.2. *Clostridium spp.*

Diarrhea can also be caused by *Clostridium* spp., which are large gram positive rods, spore-forming and strict anaerobes, being also part of the pig gut commensal microbiota and thus widely spread among swine producing areas (Songer *et al.* 2000). *Clostridium perfringens* causes type A and type C enteritis (CpA and CpC, respectively), mostly in piglets up to 14 days of age. The alpha (in CpA, CpC) and beta toxin (in CpC only) of *C. perfringens* cause more or less severe brown hemorrhagic diarrhea if the commensal microbiota flora is disrupted and *C. perfringens* is able to colonize in the small intestine mucosa and necrotize intestinal epithelial cells. The severity of CpC is much worse than CpA, which is considered a commensal organism unless it overgrows, and can even cause lesions as far as the proximal colon (Martineau *et al.* 1995; Thomson 2006). However, *C. perfringens* vaccines are available for swine. *Clostridium difficile* is a commensal bacterium usually producing monomeric enterotoxin A (TcdA) and/or monomeric cytotoxin B (TcdB) (Songer and Taylor 2006). It does not colonize the colonic epithelium unless the commensal microbiota flora is disrupted and *C. difficile* can colonize unoccupied niches (Kelly *et al.* 1984). Due to its antibiotic resistance, *C. difficile* associated

disease (CDAD) is gaining on importance in suckling pigs, mostly when sows are treated with antibiotics (Songer and Taylor 2006). However, it has been found without association to antibiotic usage (Songer *et al.* 2000). Mesocolonic edema in infected pigs can be observed during necropsy examination, as well as focal suppuration in colonic lamina propria. Exudation of neutrophils and fibrin into lumen may occur (Songer *et al.* 2000). Affected piglets are treated as described before for pathogenic *E. coli* but especially piglets affected by CDAD will not thrive and are often culled (Songer and Taylor 2006). Common methods to confirm bacterial infections are anaerobic culture of organism, ELISA, RT-PCR or DNA-hybridization (King 2003).

2.2. Pig Gastrointestinal Tract Development

2.2.1. Early Postnatal Intestinal Growth and Morphology

In the immediate postnatal period, tissue growth in the gastrointestinal tract is rapid (Fan 2003). The weight of the small intestine increases by up to 80% during the first three days of postnatal life (Xu *et al.* 1992). It has also been shown that the feeding of colostrum increases intestinal weight, surface area and brush-border enzyme activities (Wang and Xu 1996; Zhang *et al.* 1997). The intestinal mucosa consists of four layers: a monolayer of columnar intestinal epithelial cells (IEC) which overlay the lamina propria and which in turn consists of subepithelial connective tissue and lymphoid tissue with most important immunological function (Audus *et al.* 1990). The lamina propria is connected to the muscularis mucosae, a thin layer of smooth muscle, followed by the submucosa (Pearson and Brownlee 2005). Over the entire gastro-intestinal tract, specialized goblet cells are interspersed among the epithelial cells and secrete mucus in varying amounts depending on the immune status and the purpose of the tissue (Allen *et al.* 1986; Atuma *et al.* 2001). The mucus layer consists of mucins which are glycoproteins

with a protein backbone consisting of approximately 80% of carbohydrates which impart the principal viscous and gel-forming properties (Bell *et al.* 1984). The major function of mucins is to present recognition molecules similar to epithelial cell surface to provide pseudo-cell surface attachment sites for microbes (Allen *et al.* 1998; Pearson and Brownlee 2005) and to entrap nutrients in close proximity to the absorptive site in the small intestine (Acosta 2009). The mucus layer is subdivided into 2 layers: the enterocyte adherent or 'unstirred' mucus layer and the non-adherent or 'sloppy' mucus layer which is loosely attached to the unstirred mucus layer. Gastric goblet cells are concentrated in pits to prevent the enterocyte from self-degradation and to avoid adherence of bacteria to the epithelium (Laux *et al.* 2005; Pearson and Brownlee 2005). *Muc2* producing goblet cells can also be found at high counts in the intestine crypts and dispersed among the villi in the small intestine (Ouellette and Selsted 1996; Falk *et al.* 1998). The gastric pits also contain parietal cells, which are responsible for HCl and intrinsic factor secretion, as well as chief cells for secretion of pepsinogen precursor, and endocrine and neuroendocrine cells. In the intestine, pits or crypts contain goblet and paneth cells instead of parietal and chief cells. Paneth cells are located at the base of the crypt, secreting lysozyme and a variety of antimicrobial defensins (Ouellette and Selsted 1996; Falk *et al.* 1998). Stem cells are located medially along the wall of the crypts and migrate while differentiating either towards the base of the pit or up towards the neck and beyond to form the GIT enterocyte (Fan *et al.* 2001; Pearson and Brownlee 2005). The majority of the epithelial cells lining the small intestinal lumen are enterocytes capable of digesting and absorbing nutrients.

Weaning of piglets at an early age is widely practiced in the modern pig industry to maximize productivity of sows and the utilization of farrowing facilities (Maxwell and Carter 2001). Weaning age was continually moved forward during the 1990's, such that piglets are now

routinely weaned between 21 to 35 days of age in large production units (Lallès *et al.* 2007). European weaning ages (generally 28 days of age or older) are based on predicted animal welfare since early weaning of piglets is often associated with gastrointestinal disorders and retarded growth (Cranwell and Moughan 1989). However, weaning, regardless of animal age, increases the relative weight, diameter and length of the small intestine, but does not change the protein to DNA ratio of the organ (Lackeyram 2003). The observation of increased relative growth rate of the small intestine following weaning is supported by reports of increased crypt depth in neonatal pigs in response to weaning associated with increased enterocyte proliferation, but reduced villus height associated with increased apoptosis and combined increased epithelial cell turnover rates (Fan 2003). This suggests an overall high nutrient demand to maintain the intestine of the newly weaned pig. The length of time it takes piglets to adapt to weaning is variable. McCracken *et al.* (1999) concluded that piglets weaned at 3 weeks of age were able to resume normal physiological status 4 days post-weaning. However, Lackeyram (2003) showed that piglets weaned at 7 to 10 days of age did not recover from weaning associated intestinal mucosa damage until 11 to 14 days post-weaning. Age at weaning and the composition of the post-weaning diet appear to be main factors that determine the length and period of adaptation (Fan 2003; Lallès *et al.* 2007).

2.2.1. Development of Nutrient Absorption and Barrier Function

The intestinal mucosa mediates important absorptive and excretive functions. It is exposed to the non-sterile external environment, forming a barrier to the essentially sterile internal environment. Although the intestinal epithelium is only one cell layer thick, rapid turnover, tight junctions between cells, production of mucins, and secretion of anti-microbial defensins make it a relatively impermeable barrier for microbes, but the preferred site of nutrient absorption (Pearson and Brownlee 2005). The presence of intracellular tight junctions, which join epithelial

cells, help to form an important barrier, prevent the free mixing of luminal antigenic material with the mucosal immune system in the lamina propria (McCormick 2005). These tight junctions are dynamic structures that control the passive permeation of solutes through the paracellular space using proteins and signaling molecules (McCormick 2005). Gastrointestinal secretions, including the secretion of mucins, provides a non-immune line of defense, regulates luminal pH, recycles water and essential nutrients and participates in various stages of nutrient digestion and absorption (Fan 2003). The mucus layer consists of gel forming, secreted mucins derived by host expression of the *Muc2*, *Muc5AC* and *Muc6* genes which are secreted by the stomach goblet cells. Trans-membrane mucins are produced by IEC derived by host expression of genes *Muc1*, *Muc3*, *Muc4*, *Muc13*, *Muc15*, *Muc17* and *Muc20* in the small intestine, as well as additional *Muc11* and *Muc12* genes within large intestinal epithelial cells. A wider variety of mucins are secreted in the hindgut presumably in order to create a stronger barrier due to the larger commensal bacteria load (Pearson and Brownlee 2005). The attached mucin layer has been determined to be thickest in stomach and large intestine compared to the small intestine (Atuma *et al*, 2001). Though little is known about the ontogeny of the mucus secretion in the pig, it has been shown that there are clear compositional changes in the mucus secretion associated with gastrointestinal maturation (Turck *et al.* 1993). Widdowson et al. (1976) determined that at birth the piglet has some goblet cells which are ready for discharge and some which are immature. A large proportion of those cells in the duodenum discharge and are replaced by a new generation of goblet cells within 24 h of birth (Widdowson et al. 1976). Similar changes take place much more slowly (up to 10 days of age) in the large intestine (Widdowson et al. 1976).

Nutrient digestion in the small intestine requires a well-differentiated enterocyte which possesses a specific apical membrane (brush border membrane) and expresses various hydrolytic

enzymes and nutrient transporters. The brush-border digestive enzyme activity gradually increases with maturation of enterocytes along the crypt-villus axis (Fan *et al.* 2001). In addition to the progressive differentiation along the crypt-villus axis, the intestinal enterocyte also undergoes an age-related maturation process. The enterocyte has a brush border sucrase activity in fetal pigs before birth. Brush-border lactase and peptidase activities are detectable in fetal pigs at 7 weeks of gestation and reach a peak level at the time of birth (Buddington and Malo 1996). The ability to absorb macromolecules, including immunoglobulin increases rapidly during the prenatal period and reaches its peak level by birth (Sangild 2001). The activity of brush-border sucrase, maltase and maltase-glucoamylase are very low in newborn pigs (Cranwell 1995). Conversely, the brush border lactase activity reaches the highest level at the time of birth and milk lactose is well digested by suckling piglets (Cranwell 1995). The intestinal enterocyte expresses a high level of monosaccharide transporter activities at the time of birth and can absorb monosaccharides very effectively (Puchal and Buddington 1992). Suckling pigs can digest lactose well, but they have a limited capacity to digest other disaccharides and polysaccharides. The level of enterocyte brush-border aminopeptidase N activity is relatively high in suckling pigs and the activity continues to increase up to the age of weaning.

2.3. Maternal Influences on Neonatal Development

2.3.1. In Utero Influences on Postnatal Development

Coinciding with the latest advances in genome sequencing, the awareness of non-genetic factors influencing gene expression is rising. Epigenetics is the study of heritable changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence (Bird 2007). Breeding, meaning genetic selection and prediction of characteristics of the offspring, is already extremely complex and hard to predict (Henderson 1975; Tholen *et al.*

2003). Additionally, the variety of epigenetic mechanisms which can affect genetic information have increased the variability of expression of genetic material. While the connection of nutrition and health due to epigenetic factors is mostly unknown, there is research evidence which strongly correlates these factors to genetic variability both prenatal and postnatal. One of the first examples of epigenetic effects where environment and nutrition during gestation affected histone acetylation/DNA methylation resulting in long term changes in gene expression patterns was from the research on the "Dutch Hunger Winter" which lasted from November 1944 to April 1945 (Hazani and Shasha 2008). This epidemiological survey of army recruits in Holland in 1976 showed that there was a significantly higher rate of obesity and hypertension among those who had been born 20 years earlier during or just after the famine (Osmond *et al.* 1993). Prenatal hunger, particularly in the first trimester resulted in significantly increased cardiovascular morbidity, a higher incidence of asthma, an atherogenic lipid profile and glucose intolerance in as much as 21% of the adults (Lopuhaä *et al.* 2000; Roseboom *et al.* 2000). This study initiated the hypothesis that maternal environmental factors affecting gene expression patterns in the offspring through in utero modulation of histone acetylation and DNA methylation. In the postnatal period, discoveries connecting tumor cell mediated hyper-methylation of DNA and nutrition show that there are interactions between DNA methylation and nutrition that have not been fully elucidated (Huang 2002).

2.3.2. Composition of Porcine Colostrum and Milk

Colostrum provides energy for the maintenance of body temperature and normal physiological functions, as well as source of antibodies for passive immunization (Huo *et al.* 2003). The energy content of sow milk is 5.4 kJ/mL, major sources of energy being fat (60%), lactose (22%) and protein (18%; Hartmann and Holmes 1989). Porcine milk composition changes due to the nutritional needs of the offspring's stage of development (Xu 2003). Darragh

and Moughan (1998) determined that colostrum has a very high dry matter (DM) content of 24.8% compared to 18.7% in mature sow milk. The protein content in colostrum is extremely high with 60.9% of DM, declining in mature milk to 29.4% DM (Darragh and Moughan 1998). Within the protein fraction, the composition changes as well: The whey protein content of 91% of total protein in colostrum, consisting of IgG (64.8% of whey protein), IgA (10.7%), IgM (6.2%) and serum albumin (14.4%), is reduced to 52% of total protein in mature milk, consisting of IgG (3.0% of whey protein), IgA (22.2%), IgM (5.1%) and serum albumin (15.6%). Values are based on data and calculations from previously published data (Klobasa *et al.* 1987; Xu 2003). The combination of the five most prevalent limiting amino acids in sow milk, namely lysine, methionine, threonine, phenylalanine and valine, is reduced from 23.2% to 20.7% total amino acids, whereas the combination of the two most prevalent non-essential amino acids, namely glutamic acid and proline, increase from 27.2% to 33.3% of total amino acids in colostrum vs. mature milk, respectively (Csapó *et al.* 1996). The fat content increases from 23.8% DM in colostrum to 40.6% DM in mature milk (Darragh and Moughan 1998) and the fatty acid composition can directly be influenced by diet (Hartmann and Holmes 1989). On an as-fed basis, the fat content of sow colostrum and milk varies minimally around 6%, but peaks on day 3 past partum with 13%, boosting the piglet's growth (Csapó *et al.* 1996). Colostrum and milk contain negligible amounts of medium chain fatty acids and mostly long chain fatty acids (Csapó *et al.* 1996). Also the pattern of major fatty acids does not change between colostrum and mature milk, but the concentration of oleic acid (18:1) and linoleic acid (18:2) is higher in colostrum being 37.5% and 12.7% of total fatty acid methyl esters, respectively, compared with mature milk being 32.7% and 10.0% of total methyl esters, respectively (Csapó *et al.* 1996). This provides the neonatal pig with relatively higher levels of essential fatty acids in colostrum

compared to mature milk. The increase in lactose from 13.7% DM to 28.3% DM, respectively (Darragh and Moughan 1998) is the driver for the higher water content in mature milk, accommodating the higher feed intake potential and the increased need for fluid of the piglet. Total mineral content of 2.8% DM in colostrum increases to 4.3% DM in mature milk (Darragh and Moughan 1998), reflecting the mineral need of the growing pig for bone development. The generally higher vitamin content in colostrum compared to mature milk again shows the promotion of growth of the new born piglet (Csapó *et al.* 1996).

2.3.2. Contribution of Colostrum and Milk to GIT Development

The development of the new born piglet is majorly influenced by the sow in regard to sufficient nutrition, protective factors within maternal milk and passive immunization as described in the previous section. These factors are primarily associated with immediate impacts on growth and GIT development. Sow milk contains a number of infection preventing agents. Lactoferrin has a high affinity to bind iron (Goldman and Goldblum 1989) and is resistance to protease degradation. Its receptor mediated uptake from the gut gives the piglet an advantage in iron absorption after birth (Gíslason *et al.* 1993). Additionally, the mammary glands of the sow secrete neutrophils and macrophages capable of phagocytosis, as well as pH stable milk lymphocytes, all of which help in pathogen recognition and elimination in the gut lumen (Robinson *et al.* 1978; Skansen-Saphir *et al.* 1993; Wuryastuti *et al.* 1993). Concentrations of hormones secreted in sow's milk, namely epidermal growth factor (EGF), insulin and insulin-like growth factors (IGF-I, IGF-II) and transforming growth factor-beta (TGF-β), are high in colostrum and decline rapidly within 48 h after birth. They generally aid gastro-intestine tract (GIT) growth and maturation after birth or/and prevent immune response (Carpenter 1980; Ishizaka *et al.* 1994; Xu *et al.* 1994; Xu 1996). Acquired passive systemic immunity is provided to the piglet through the absorption of maternal IgG and IgA (Fan 2003). Even though the level

of immunoglobulins such as IgG decrease dramatically within the first 48 h past partum, IgA contents in mature sow milk are still remarkably high (Hartmann and Holmes 1989). Immunoglobulin A is blocking potentially pathogenic microbiota from epithelial cells, similar to a wide variety of oligosaccharides and glycoproteins in sow colostrum and milk, either competing for host epithelial cell receptors or presenting host cell surface markers themselves (Goldman and Goldblum 1989). The combination of all these protective agents from the sow is additionally needed to ensure proper gut closure once the piglet is able to synthesize its own antibodies (Fan 2003).

2.4. Microbial Influences on Pig GIT Development

2.4.1. Microbial Sensing in the GIT

Microfold or Membranous (M-) cells are responsible for antigen sampling in the small and large intestine (Brandtzaeg and Pabst, 2004). In the small intestine, M-cells are mainly found in Peyer's Patches (PPs) as well as in Isolated Lymphoid Follicles (ILFs) whereas, in the large intestine, M-cells are part of ILFs only (Brandtzaeg and Pabst, 2004). M-cells in Peyer's Patches of the small intestine have been shown to express Human Leucocyte Antigen (HLA)-class II antigens, which are important in tolerance induction to commensal microbiota (Ueki *et al.*, 1995). However, M-cells in ILFs express Intracellular Adhesion Molecule 1 (ICAM-1), which is believed to play a role in inducing inflammation of colonic mucosa (Ueki *et al.*, 1995). Microfold cells are able to present luminal antigens to underlying antigen-presenting cells (APCs). These APC's are able to distinguish pathogenic microbes from commensals by the expression pattern recognition receptors (PPRs) which recognize particular microbial components (Ventura *et al.* 2012). Pattern recognition receptors include the transmembrane Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) which recognize and bind

microbial associated molecular patterns (MAMPS) from gut microbiota (Kawai and Akira 2011). Lamina propria CD11c+CD11b+ dendritic cells are a very important APC, which mediate naïve B-cell maturation to IgA producing plasma cells (Uematsu *et al.* 2008). Antigen-presenting cells therefore play a pivotal role in the interaction between intestinal microorganisms and host immune defense, immune-cell recruitment, IgA production, and mucosal homeostasis (Kawai and Akira, 2011).

In intestinal epithelial cells, Toll-like receptors 2 and 4 (*TLR2*, *TLR4*) play a critical role in host detection and response to Gram-positive and Gram-negative bacteria, respectively (Figure 2.1). Following stimulation of *TLR2* and *TLR4*, the major signaling pathway is activated, recruiting TRAF6 via TIR adapter Protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88), which mediates activation of nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) and NFκB inhibitor α (IκBα) protein complex (Kawai and Akira, 2007). Proteins IκBα and NFκB are derived by expression of *NFKBIA*, *NFKB1* (tightly regulated) and *NFKB2* (loosely regulated) genes (Hay *et al.*, 1999). Upon degradation of IκBα, NFκB is able to translocate to the nucleus and induce inflammatory cytokine gene expression (Hay *et al.*, 1999; Kawai and Akira, 2007). Pro-inflammatory interleukins (IL) are expressed, which regulate expression of death ligand Tumor Necrosis Factor α (*TNFα*), Proliferation Cell Nuclear Antigen (*PCNA*) and mucin (Muc) gene expression (Roth *et al.*, 1995; Deplancke and Gaskins, 2001). In enterocytes, *TLR4* is also able to recruit TRAF3 via TIR-domain containing adapter inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM), which mediates Interferon Regulatory Factor 3 (*IRF3*) phosphorylation, allowing *IRF3* dimers to translocate to the nucleus (Kawai and Akira, 2007). In the nucleus, *IRF3* dimers induce

interferon (IFN) gene and Fas Ligand (*FasL*) death ligand expression (Ruemmele *et al.*, 1999; Kawai and Akira, 2007).

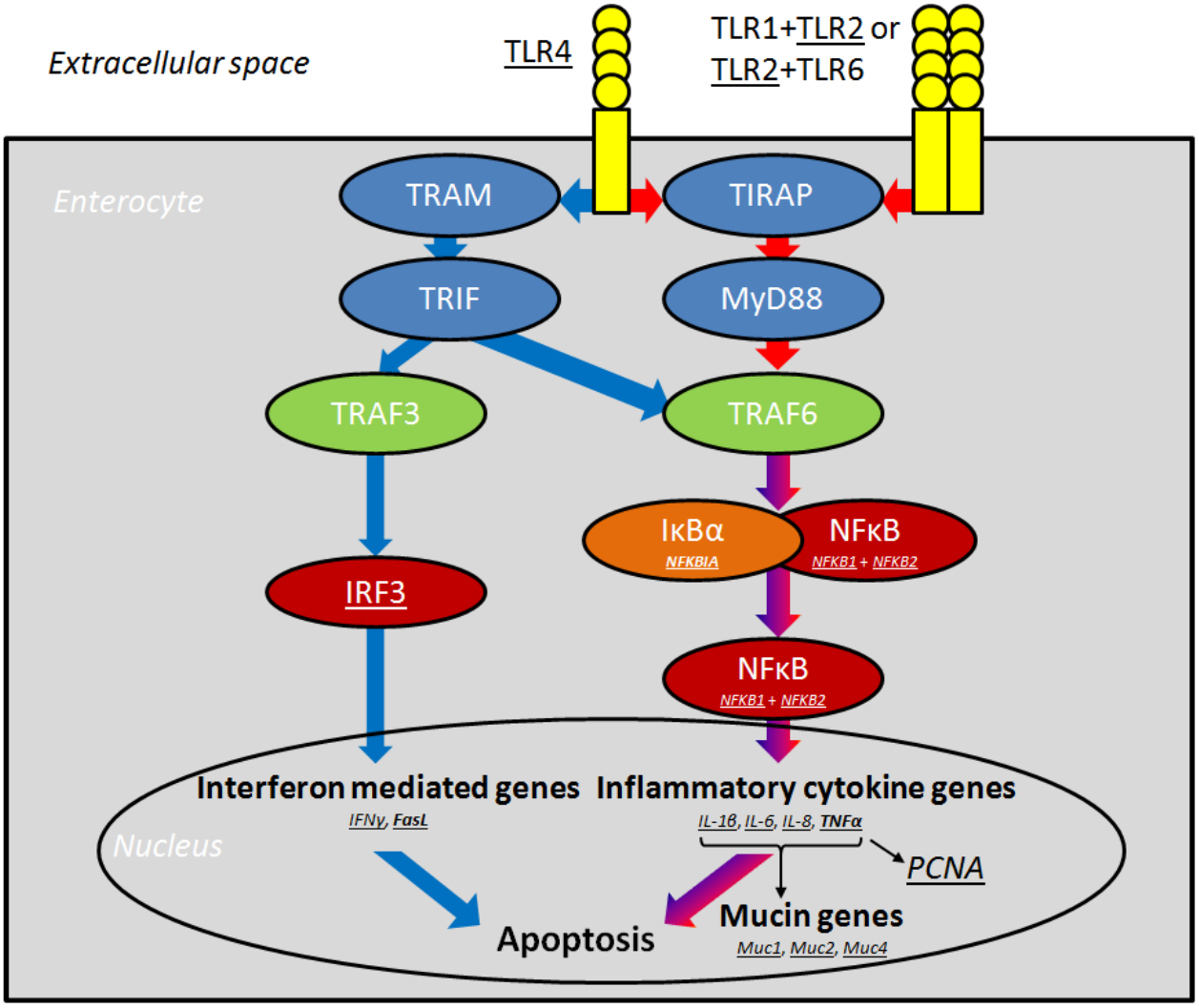

Figure 2.1. TLR signaling adapted from Kawai and Akira (2007) with findings from Roth *et al.* (1995), Ruemmele *et al.* (1999), and Deplancke and Gaskins (2001). Adapters with Toll-Interleukin-1 Receptor (TIR) domain in blue, TNF Receptor Associated Factor (TRAF) adapters in green, mediators capable of translocation to the nucleus in red, inhibitory protein in orange; Underlying genes are italicized if the name differs from the protein; Genes measured in chapters 6 and 7 are underlined.

2.4.2. Microbial Influences on GIT Function

Physiological effects microbial colonization on the host not only includes development of the adaptive immune response, but many parameters of innate immunity, digestive function, metabolism of absorbed nutrients and even systemic effects (Tanida *et al.* 2005). Early reports comparing germfree and conventional animals showed many physiological differences in the host including microbial dependent changes in organ weights and intestinal structure (Gordon *et al.* 1966; Wostmann 1981). More recent investigations have begun to examine the host-microbial interaction at a closer level using molecular (Shirkey et al. 2006; Willing and Van Kessel, 2007), genomic (Chowdhury *et al*. 2007) and proteomic (Danielsen *et al.* 2007) techniques combined with gnotobiology to define the physiological effects of a single or groups of organisms on specific cell populations in vivo.

The response to a single organism, *Bacteroides thetaiotaomicron*, leads to differential transcription of genes responsible for nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis and intestinal maturation (Hooper *et al.* 2001). A similar response observed in zebra fish to the same organism indicates an evolutionary conservation of host-microbial interactions (Rawls *et al.* 2006). *L. reuteri* has been described to induce dendritic cell mediated self-specific host tolerance as demonstrated in specific *Lactobacillus* free mice (Hoffman *et al.*, 2008; Livingston *et al.*, 2010). Upon introduction and initial detection of *L. reuteri*, pro-inflammatory cytokines were temporarily up regulated but rapidly declined in spite of high levels of *L. reuteri* in the murine gut after 21 days.

In contrast to host pathways designed to recognize and orchestrate appropriate host responses to microbiota, bacteria can produce specific effector molecules whose function is to regulate targeted host functions to benefit the bacteria. The use of effector molecules by pathogenic bacteria has been intensely studied and serves a variety of functions in pathogen establishment,

while relatively little is understood about effector molecules secreted by commensal bacteria. Commensal bacteria secrete effector molecules that have been shown to stimulate the host to provide a nutrient source, as well as inhibit the inflammatory response (Hooper and Gordon 2001a,b; Sougioultzis *et al.* 2006). For example, *Saccharomyces boulardii* reduces the inflammatory response by secreting a small heat stable, water soluble factor that blocks nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα, derived via expression of *NFKBIA*) degradation thus reducing nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) binding to DNA, and subsequently reduces interleukin-8 (*IL-8*) expression (Sougioultzis *et al.* 2006). Similarly to the factor secreted by *S. boulardii*, butyrate is the best known microbial anti-inflammatory metabolite. It equally inhibits NFκB complex activation by preventing IκBα degradation and at the same time prevents luciferase gene expression in human and rodent colonic cells (Segain *et al.* 2000). Other bacteria with anti-inflammatory qualities form *Streptococcus* and *Bifidobacterium* genera have been reported to prevent NFκB complex degradation due to bacterial metabolites as well (Ménard *et al.* 2004). Differing from that, *Faecalibacterium prausnitzii* was determined to express anti-inflammatory metabolites reducing *IL-8* gene expression in Caco-2 cells (Sokol *et al.* 2008). In addition, an increase of anti-inflammatory *IL-10* gene expression due to *F. prausnitzii* supernatant supplementation was observed in disease challenged colonocytes of mice in the same study (Sokol *et al.* 2008). Both effects were determined to be independent of butyrate production of *F. prausnitzii*.

2.4.3. Effects of Commensal Microbiota on the Host

Commensal bacteria are capable of inhibiting inflammation (Kelly *et al.* 2004) and outcompeting pathogens (Freter 1974; Hudault *et al.* 2001), making the commensal microbiota the first line of defense of the host. In addition to the defenses of the commensal bacteria, tight

junctions between enterocytes begin to form at birth, however, bacterial colonization leads to a much stronger barrier by enforcement of tight junctions. Tight junction protein, zona occludens 1 (ZO-1), is also up regulated by bacterial recognition (Cario *et al.* 2002). The stronger barrier results in a more stable electrolyte gradient and thus supports passive absorption of minerals and mineral co-transported nutrients like glucose. Mucin secretion plays an important role in host defense and is also highly affected by bacterial colonization (Hecht 1999), for example *Lactobacillus casei* increases secreted mucin gene expression of *Muc2* in intestinal epithelial cell monolayers as compared to untreated cells (Mattar *et al.* 2002), while other bacteria have been shown to increase the expression of both secreted *Muc2* and transmembrane *Muc3* by goblet cells (Mack *et al.*1999; Mack *et al.* 2003).

2.5. Evidence for Long Term Effects of Early Postnatal Environment

2.5.1 Immunologic Mechanisms

As previously stated (Section 2.4.1), the mucosal immune system serves as a major mechanism providing protection against pathogens involving gut-associated lymphoid tissue (GALT), also called Peyer's Patches. However, the immune system must first be primed by exposure to antigens and other exogenous stimuli which then activate microbial sensing via TLRs (Bauer *et al.* 2006). This has been supported over multiple studies with preterm infants showing overall low circulatory IgA with increased circulatory IgM being the only response to infection (Stoll *et al.* 1993). This contrasts full term infants, also with low initial amounts of circulatory IgA, but which quickly increased due to exposure to commensal bacteria and pathogens (McKay and Thom 1969; Nahmias *et al.* 1991). In intestinal mucosa, secretory IgM is the most predominant immunoglobulin of the host for approximately one month after birth. More stable secretory IgA can be detected after approximately 10 days post-partum and becomes the

predominant secreted immunoglobulin after one month (Crabbé *et al.* 1970; Brandtzaeg, 2010). This indicates that commensal bacteria may be critical in maturation maturation host immunity. The research has led to the hygiene hypothesis which is based on the assumption that altered microbial diversity and functional activities of the luminal microbiota due to lack of early exposure to infectious agents may be linked to the etiology of immune-mediated diseases at later stages of life (Kelly *et al.* 2007).

Although the precise mechanisms are unknown, research demonstrates that the commensal gut microbiota can generally influence epithelial and stromal cell biology, as well as the function of dendritic, T- and B-cells. This suggests that, at the level of the mucosal immune system, the effects of commensal gut microbiota are profound and long lasting (Kelly *et al.* 2007). For example, a single bacterial polysaccharide from a commensal bacterium, *Bacteroides fragilis*, is capable of directing the cellular and physical maturation of the immune system by producing a zwitterionic polysaccharide (ZPS) that can correct certain immune dysfunctions (Mazmanian and Kasper, 2006). This was supported by research in rodents simulating inflammatory bowel disease and allergic asthma. It was shown that exposure of commensal bacteria to neonatal but not adult germ-free mice reduced accumulation of invariant natural killer T (iNKT) cells in colon lamina propria and lung and associated symptoms in tested animals (Wei *et al.* 2010; Olszak *et al.* 2012). It was demonstrated that gut microbiota in general play a key role in autoimmune disease stimulation and diabetes type 1 symptom development (Wen *et al.* 2008; Berer *et al.* 2011), however, *Bifidobacterium pseudocatenulatum* was shown to exhibit anti-allergic effects *in vitro* (Kasakura *et al.* 2009) and that increased perinatal supplementation of pre- and probiotics can delay the onset of allergies past two years of age (Kukkonen *et al.* 2010).

Due to those and other effects, host gut commensal bacteria genera have been categorized into either harmful or beneficial microbes (Gibson and Roberfroid, 1995). Harmful bacteria like sulfate reducers, *Vibrio, Staphylococcus* and *Pseudomonas* were associated with diarrhea, infections, toxin production, putrefaction and constipation, beneficial genera like *Lactobacillus* and *Bifidobacterium* were associated with colonization resistance and competitive exclusion, immunomodulation and improved host development, modulation of blood lipids, improved hormonal regulation and nutrient uptake of the host. Enterobacteria, *Enterococcus* and *Bacteroides* microbiota groups could not be clearly associated with a healthy gut development since there were determined to contain both, beneficial and harmful representatives (Gibson and Roberfroid, 1995). However, *Bacteroides thetaiotaomicron* is a commensal microbe known for its ability to induce fuccosylation (Hooper *et al.* 2001). This ability could affect the mucus environment and thus colonization by other bacteria. Although *Clostridium* was described as being harmful in Gibson and Roberfroid (1995), this would mostly account for proteolytic *Clostridium* cluster I with its predominant commensal representative *Clostridium perfringens*. Research in pigs and humans has indicated that fiber degrading *Clostridium* cluster IV and XIVa are beneficial for the host (Barcenilla *et al.* 2000; Castillo *et al.* 2007).

In general, it was determined that successive and differing populations of bacteria colonizing the GIT not only contribute to host immune system development, but also to the development in nutrient digestion (Suzuki *et al.* 2007). Dinan *et al.* (2010) summarized that commensal bacteria have profound effects on immunological differentiation in host development and emphasized that the immune system is structurally and functionally impaired without gut microbiota.

2.5.2. Epigenetic Mechanisms

The connection of nutrition and health due to epigenetic factors has been strongly correlated to genetic variability resulting histone acetylation/DNA methylation during gestation (Section 2.3.1) as well as during the postnatal period. Nutrition during infancy has been hypothesized to cause metabolic imprinting of GI structure and function by interacting with developmental epigenetics based on the assumption that epigenetic pathways are involved in GIT development and maturation (Waterland, 2006). Fibrolytic gut microbiota like *Clostridium* cluster IV and XIVa have been determined to produce butyrate from digestive fiber in the mammalian hindgut (Bergey and Holt, 1994; Barcenilla *et al.* 2000) which is then directly involved in a well described epigenetic pathway: histone acetylation. Kien *et al.* (2006, 2008a, 2008b) determined that butyrate production by gut microbiota in the cecum of pigs has a direct impact on crypt cell proliferation, via inhibition of histone deacetylase enzyme complexes which reduce transcription (Boffa *et al.* 1978).

More recent research concluded that commensal bacteria epigenetically modify the host genome in non-crypt IEC by methylation of toll-like receptor 4 (*TLR4*) gene and histone deacetylation in order to mute the immune response to commensal microbiota (Takahashi *et al.* 2009, 2011). Although the underlying mechanisms are not completely understood, it was determined that *TLR4* gene expression in non-crypt IEC is regulated by lipopolysaccharide (LPS) substrate.

Another powerful mechanism of epigenetic regulation is the influence of early nutrition on CpG methylation through its effects on mammalian 1-carbon metabolism (Waterland 2006). This system provides the methyl groups for the methylation of DNA and various other biological substrates, and is dependent on diet-derived methyl donors including folate, vitamins B12 and B6, choline, and methionine (Van den Veyver 2002). Although those ingredients are mostly

supplemented to modern pig diets in pure chemical form, one could argue that those nutrients, except for choline, are or could be derived by microbial fermentation (Mondal *et al.* 1996; Martens *et al.* 2002; Zhu *et al.* 2005; Shimizu, 2008). Dietary deficiency or excess in some of these important nutrients could affect the efficiency of 1-carbon metabolic pathways and influence the stochastic establishment of CpG methylation during development. Imbalance between nutrient intakes (qualitative and quantitative), metabolites, and the precise requirements of these epigenetic processes, within critical spatiotemporal limits can lead to defective structural and functional development or even the absence of certain specialized cell types, leading to a "no return" situation because of irreversible processes of differentiation (Van den Veyver 2002).

Numerous developmentally regulated changes in gene expression have been documented in animal models during the prenatal and very early postnatal period, proving these to be critical times in development (Gluckman *et al.* 2007; Nafee *et al.* 2008). While only few examples of epigenetic influences via nutrition are known later in life (Bouret *et al*. 2004), research is starting to show the role of gut microbiota especially in the postnatal period. However, despite continual advancement in understanding epigenetic mechanisms, further research is required to understand if there are periods of development throughout maturation, when impacts on these mechanisms can be made, and how they interplay with microbial succession.

2.5.3. Microbial Succession

The human gut microbiota, shaped by the long co-evolutionary history of symbiotic host–microbe interaction, plays an important role influencing the host. The inter-relationships between the microbiota and the host are then clearly important in relation to overall health and an imbalance between these systems (infants delivered by cesarean section) results in changes in intestinal microbiota which are quite persistent (Grölund *et al.*1999). This is supported by epidemiological and clinical data, suggesting that the decreased incidence of infections observed

over the last three decades, particularly in westernized societies, may predispose to diseases such as type I diabetes mellitus, autoimmune diseases, as well as atopic and inflammatory diseases especially of the bowels (Björksten *et al.*2001; Murray *et al.*2005; Kelly *et al.*2006; Ott and Schreiber 2006).

As stated by Kelly *et al*. (2007), the hygiene hypothesis assumes that altered microbial diversity of the GIT microbiota, due to lack of early exposure to infectious agents, may be linked to immune-mediated diseases throughout life. Gut microbiota of the suckling pig starts stabilizing within several days of age (Conway 1996) but is still variable to some degree (Kelly 1998). It has been indicated that the best opportunity to impact the gut microbial community is early in the postnatal period, prior to the establishment of the core microbiota (Kelly *et al.* 2007). Research has shown that despite a large degree of variation between co-housed and related individuals in the gut microbial phylogeny, there is a large array of shared microbial genes among researched individuals (Thompson *et al.* 2008; Turnbaugh *et al.* 2009). This indicates that with the introduction of a microbial population in a niche, their presence is maintained if there is an open role within the core "gene" microbiota (Turnbaugh *et al*. 2009). Once a microbiota is established in an environmental niche in the gut, it needs drastic life events like weaning or antibiotic treatment, to alter microbiota composition (Kelly 1998).

The mechanisms by which commensal/symbiotic bacteria contribute to immune development, immune homeostasis and regulation, are now being actively researched and debated. However, despite a lack of understanding, due to the potential beneficial effects of commensal bacteria on immune function, there is now considerable interest in using probiotics for the treatment of a wide range of disorders (O'Hara and Shanahan 2007). By promoting a 'healthy gut microbiota' in early life through appropriate microbial colonization (environmental

exposure), nutrition and possibly through the use of new probiotics, mucosal immunity and natural resistance to infections may be enhanced and could potentially have long lasting effects.

2.6. Conclusions

Gut microbial succession after birth is crucial for host development. Many factors impact the taxonomic pattern of microbial succession, like host genotype, maternal influences pre- and postnatal, diet, exposure to pathogens, antibiotic treatment. Parallel to host microbiota development, host gut tissue maturation occurs. The capacity of the small intestine to digest and absorb complex nutrients as well as immunological functionality increases over time and in constant interaction with the gut microbiome. There are many studies suggesting that changes in neonatal environment have long term impacts on the pig's digestive physiology. However, there is no conclusive evidence proving the hygiene hypothesis concept. Reported evidence suggests it is likely that the taxonomic microbial succession pattern has long term impacts on microbial ecology and GIT function.

2.7 Hypotheses and Objectives.

Based on the above review of the literature, we hypothesized that alterations in the early postnatal microbial succession pattern in intestine impact post weaning intestinal microbial ecology and/or host responsiveness to the intestinal microbiota (the host:microbial interface) affecting growth and nutrient assimilation. The objectives of the first experiment (Chapters 3 and 4) were, to first profile early postnatal bacterial succession patterns in intestine of the conventional pig using culture-independent methods and secondly, to identify potentially beneficial and harmful commensal bacterial candidates for future gnotobiotic trials. Although there has been substantial previous work assessing microbial succession in the pig, the very early

succession profile was not clearly established; information considered important in linking this period postweaning responses. Chapter 3 focused on profiling succession in intestinal contents primarily at the genus level and in both proximal and distal regions. Since community structure analysis presented in Chapter 3 indicated microbial communities formed two groups based on age (0.25 to 2 d of age and 3 -20 days of age), further work added detail to succession patterns (Chapter 4) by comparing community changes between 2 and 20 days of age at the species level in multiple locations and in both contents and mucosa. The objectives of the second experiment in Chapter 5 were to establish a model by which the early postnatal microbial succession pattern in intestine could be modified and to determine whether succession modification altered post weaning microbial ecology and host growth performance. The "snatch farrow" and isolator rearing model established in the second experiment resulted in surprisingly limited differences in fecal microbial ecology after 4 days of isolator rearing and no detectable changes in postweaning ecology. The model was also technically challenging and thus abandoned in favour of the model reported in Chapters 6 and 7. In the last two experimental chapters a gnotobiotic model was employed in which germ-free pigs were derived by caesarian section and monoassociated with selected bacterial species based on data presented in chapters 3 and 4. The model permitted specific control of early colonization and permitted association of any observed responses with the specific monoassociated bacterium. In chapters 6 and 7 monoassociation with different bacterial species during the first 4 postnatal days was hypothesized to alter the postweaning microbial profile and alter the host:microbial interface affecting growth performance and nutrient assimilation pathways. Three mechanisms were postulated to mediate postweaning changes in ecology and the host:microbial interface. Firstly, the monoassociated bacterium could be advantaged in establishing a microbial niche due to prolonged colonization without competition.

This colonization advantage, prior to exposure to complex environmental organisms, could have had cascading effects on the succession and structure of the entire complex microbial community. Changes in postweaning community composition could impact host immune response, mucosal physiology and growth rate. Secondly, we postulated that sensing of the different monoassociated species in the first 4 days of life through, for example, the TLR pathway, could alter postweaning TLR signaling, inflammatory response and epithelial function. Finally, monoassociation during days 0-4 of age, could affect specific immune mechanisms leading to activation of tolerance or immunity and thus shaping the subsequent microbial community composition. Chapter 6 examines this hypothesis in small intestine and Chapter 7 in colon.

3.0. QUALITATIVE AND QUANTITATIVE DETERMINATION OF MICROBIAL SUCCESSION IN THE GASTRO-INTESTINAL TRACT OF THE PREWEANED PIG

3.1. Abstract

Microbial succession in the gastro-intestinal (GIT) tract of the neonatal commercial suckling pig was investigated by constructing 16S rRNA gene sequence libraries and quantitative polymerase chain reaction (qPCR) with 16S rRNA gene specific bacterial group primers using genomic DNA extracted from digesta collected along the GIT. Collection of digesta at locations between stomach and colon of 48 sow-reared piglets from 6 litters was done periodically over 20 days. Sequence libraries were constructed, revealing that *Clostridium perfringens* were most prevalent in the GIT microbiota of the neonatal pig up to day 0.5 of age, accompanied by a high abundance of *Escherichia* and *Shigella* spp. On day 1 of age, *Streptococcus* spp. became most prevalent. Between days 3 and 20 of age, *Lactobacillus* spp. were the most abundant bacteria identified. *Bacteroides* spp. were recovered in low abundances as early as 2 days of age. Major trends in bacterial succession were similar based on sequence representation in libraries and qPCR enumeration. Distinct variations between proximal and distal GIT locations were observed, including increasing abundance of 16S rRNA gene copies enumerated towards the distal gut. There was no evidence for an effect of litter of origin on microbial composition. This study has established a detailed microbial succession pattern along the entire digestive tract in the healthy suckling commercially reared pig, including the periods that may be critical for colonization of potentially harmful (*Clostridium* and *Veillonella* spp.) or beneficial, (e.g. *Lactobacillus* and *Bifidobacterium* spp.) bacteria.

3.2. Introduction

Microbial exposure early in life of a piglet has a significant impact on both adult gut microbiota composition and host-microbiota interactions which can potentially have long term implications on animal health and productivity (Inoue *et al.* 2005; Tannock 2005). Detailed knowledge of the microbial succession pattern in the commercial pig, particularly in the upper GIT, remains limited despite numerous studies which have used both culture and non-culture-based approaches (Ducluzeau 1985; Fuller *et al.* 1978; Gancarčíková *et al.* 2008; Inoue *et al.* 2005; Konstantinov *et al.* 2006; Mikkelsen *et al.* 2003; Pedersen and Tannock 1989; Smith 1965; Swords *et al.* 1993). Using both culture (Swords *et al.* 1993) and culture-independent (Inoue *et al.* 2005; Thompson *et al.* 2008) approaches, fecal microbial analysis has shown the importance of Clostridiaceae, Enterobacteriaceae and Lactobacillaceae families in the pig. Konstantinov *et al.* (2006) extended these observations and included culture-independent analysis of succession patterns in the pig ileum. There remains, however, limited data regarding early postnatal microbial succession patterns in the pig focusing on the upper gastrointestinal locations whereas a more complete understanding is required to inform development management and nutritional practices which may be beneficial in early life. Furthermore, although maternal effects on microbial profile have been reported in rodent (Gareau *et al.* 2006) and man (Inoue *et al.* 2005) litter effects have not been identified in pigs and warrant further examination. Therefore, an experiment was conducted to more fully profile early postnatal bacterial succession patterns in the pig by sampling at multiple locations along the entire digestive tract using culture independent detection methods. The goal was to identify the development of potentially beneficial and harmful bacterial groups and species (Bourlioux *et al.* 2003) over time and in different locations in clinically healthy suckling pigs.

3.3. Materials and Methods

3.3.1. Animals and sample collection

Forty-eight piglets (PIC Camborough®, Large White x British Landrace, PIC Canada Ltd. Winnipeg, MB, Canada) from 6 litters in a standard production environment were used (Prairie Swine Inc., Saskatoon, SK, Canada). Piglets remained with the sow for the duration of the trial and were subjected to routine processing at 1 d of age. Animals were humanely killed by emersion in CO_2 and exsanguination at 0.25, 0.5, 1, 2, 3, 5, 10 and 20 d of age to permit collection of digesta from the stomach, duodenum [proximal one third of small intestine (SI)], jejunum (middle one third of SI), ileum (distal one third of SI), cecum and colon. Entire contents were removed from each segment and homogenized before subsampling for further analysis. To be able to determine a potential maternal influence on microbial succession the temporal sampling scheme was designed to control for litter of origin. Due to constraints on sampling which required euthanasia, two piglets from each of litters 1 and 2 were killed for each of the first 4 collection time points whereas 2 piglets from each of litters 3 and 4 were killed for each of the second 4 collection time points. One piglet from each of litters 5 and 6 were killed at each time point. Digesta samples were immediately frozen on dry ice and stored at -70ºC until DNA extraction. The experimental protocol was approved by the University of Saskatchewan Animal Research Ethics Board (AUP Number 20070073) according to guidelines established by the Canadian Council on Animal Care (Olfert *et al.* 1993)

3.3.2. Sequence library construction

Bacterial DNA was extracted from digesta as described by Dumonceaux *et al.* (2006). Twenty-four 16S rRNA gene sequence libraries were constructed from 3 locations [stomach (Sto), small intestine (SI) including duodenum, jejunum and ileum, large intestine (LI) including cecum and colon] at 8 time points (0.25, 0.5, 1, 2, 3, 5, 10 and 20 days of age). Samples of

individual animals were pooled based on DNA concentration, pools were then amplified using universal 16S rRNA primers F1 and R3 (Dorsch and Stackebrandt 1992), as described in Petri *et al.* (2010). Specific analysis of *Bifidobacterium* species was conducted using a pool of equal DNA concentrations of all samples and 16S rRNA gene primers reported by Matsuki *et al.* (2002). PCR reaction conditions were 1 x 95 °C for 3 min, 40 x (95 °C for 40s, annealing temperature for 40s, 72 °C for 60s), 1 × 72 °C for 3 min. Amplification was performed using 5 µL of 10xPCR buffer, 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 1 µL of 10 mM primer (each), 2 µL of 1 µg/µL of bovine serum albumin, individually pipetted 0.5 µL taq polymerase and 2 µL template DNA with 2 ng sample DNA/µL, 36 µL H_2O, 0.5 µL taq polymerase (all solutions from BioRad Laboratories Ltd. Mississauga, ON, Canada). Primer sequences and corresponding annealing temperatures used for library construction are provided in Table 3.1.

The resulting PCR products were gel purified (QIAEX II Agarose gel DNA extraction kit, Qiagen Canada Inc. Toronto, ON, Canada) and ligated into cloning vector pGEM T Easy (Promega Corporation, Madison, WI, USA). Ligation reactions were used to transform *E. coli* JM109 competent cells and cultured on Luria Bertani (LB) media with 100 µg / g Ampicillin (both Thermo Fisher Scientific, Ottawa, ON, Canada). For each library, 27 colonies (10 only for the *Bifidobacterium* spp. specific group primer) were randomly selected and the plasmid inserts sequenced (Sanger *et al.* 1977) at the National Research Council, Plant Biotechnology Institute, (Saskatoon, SK, Canada) using T7 and SP6 sequencing primers (Promega, Madison, WI, USA). Forward and reverse partial sequences ($n_{16S\ rRNA\ gene}$ = 620, approx. 650 - 700 bp; $n_{Bifido16S\ rRNA\ gene}$ = 8, approx. 350-550 bp) were assembled and primer and vector sequences removed using

Table 3.1. Primers and conditions used for PCR and qPCR reactions.

Target	Ori[1]	Sequence (5'-3')	Temp.	Size	Origin
16S rRNA gene target [2]	f r	GAGTTTGATCCTGGCTCAG TCTACGCATTTCAC	50 °C	693 bp	Dorsch and Stackebrandt 1992
V3 Domain 16S rRNA gene [3]	f r	CGGYCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	60 °C	200 bp	Lee *et al.* 1996
Lactobacillus spp. [3]	f r	GCAGCAGTAGGGAATCTTCCA GCATTYCACCGCTACACATG	60 °C	349 bp	Castillo *et al.* 2006a Walter *et al.* 2001
Clostridium cluster I spp. [3]	f r	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	63 °C	346 bp	Song *et al.* 2004
Streptococcus spp. [3]	f r	AGAGTTTGATCCTGGCTCAG GTTAGCCGTCCCTTTCTGG	57 °C	485 bp	Nübel *et al.* 1996 Franks *et al.* 1998
Enterobacteria [3]	f r	ATGGCTGTCGTCAGCTCGT CCTCATTCTTTTGCAACCCACTC	60 °C	385 bp	Castillo *et al.* 2006a
Bifidobacterium spp. [2, 3]	f r	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	56 °C	563 bp	Matsuki *et al.* 2002
Bacteroides / Prevotella spp. [3]	f r	GGTGTCGGCTTAAGTGCCAT CGGAYGTAAGGGCCGTGC	60 °C	140 bp	Rinttilä *et al.* 2004

[1] Orientation: f = forward, r = reverse; [2] standard PCR with 50 µL reaction volume for sequence libraries; [3] quantitative PCR with 13 µL reaction volume for enumeration.

PreGap4 (version 1.5) and Gap4 (version 4.10) in the Staden software package (release 2004.1; J. Bonfield, K. Beal, M. Betts, M. Jordan, and R. Staden, 2004).

High quality 16S rRNA gene sequences were classified using the Greengenes Database (http://greengenes.lbl.gov/; 4) and NAST alignment tool (DeSantis *et al.* 2006) with a standard value for minimum percent identity and an automated chimera check, as well as by using classifier and seqmatch procedure of Ribosomal Database Project (RDP; release 10; http://rdp.cme.msu.edu/; Wang *et al.* 2007) with default threshold option. The NAST alignment ($n_{16S\ rRNA\ gene}$ = 518) was inspected with ARB software (version 07.12.07org; http://www.arb-home.de; Ludwig *et al.* 2004) and corrected manually based on known secondary structure when necessary (Leser *et al.* 2002). For phylogenetic analysis, a bootstrap tree of reference strains was established using ARB Neighbor Joining Distance Matrix method with Felsenstein correction and 1,000 times resampling, then sample sequences were included using ARB parsimony without changing the original topology of the tree. For further analysis, a bootstrapped phylogenetic ARB tree containing all universal 16S rRNA gene sequences was exported with Newick tree format including branch lengths. Branches were grouped by day of age and location to allow statistical analysis of microbial community structure.

3.3.3. Quantitative PCR

Quantitative PCR (qPCR) was performed using previously described primer sets and annealing temperatures (Table 3.1); reaction conditions were: 1 x 95 °C for 5 min, 40 x (95 °C for 30s, annealing temperature 72 °C for 40s. The melt curve was determined from 65 °C to 95 °C, increment 0.5 °C for 5s. Amplification was performed in a 13.0 µL reaction mixture using 6.5 µL iQ SYBR Green Supermix (BioRad, Guénette, QC, Canada), 1.0 µL DNA sample, diluted to a concentration of 2 ng DNA/µL, 0.8 µL of each, 25 mM forward and reverse primer (Table 3.1), and 3.9 µL H_2O. Enumeration of total bacteria, *Lactobacillus, Clostridium* cluster I

(Ccl1), *Streptococcus*, *Bifidobacterium*, *Bacteroides*/*Prevotella* spp. and Enterobacteria was conducted. For analysis, a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, QC, Canada) was used. Standard curves were generated using gel purified (QIAEX II, Qiagen Canada Inc. Toronto, ON, Canada) amplicons prepared by standard PCR with pooled extracted DNA as template using primers described in Table 1 and standard PCR conditions previously described for library construction. Amplicons were quantified by $O.D._{260nm}$ and 6 serial 1/10 dilutions prepared starting with 5 pg/µl concentration and including a no DNA template control. Standards were converted to copy numbers using the formula:

Number of copies = [DNA (pg) × $6.022x10^{23}$ (copies per mole)] / [fragment length (bp) x $1x10^{12}$ (pg / g) x 650 (g / mole of bp)]

All amplifications were followed by melt curve analysis (BioRad CFX manager software, version 1.6.541.1028, BioRad, Guénette, QC, Canada) to ensure single product amplification. Duplicate threshold cycle mean values with greater than ± 0.50 standard deviations were reanalyzed. Acceptable reaction efficiency was set for the range 0.90 - 1.10, and standard curve R^2 values to $\geq$ 0.97. Results were expressed in number of gene copies per g of content and as gene copies for the target bacterial group per total eubacteria, expressed as a percent. The formulas used were: Number of copies / g of content = [Volume after DNA extraction (µL) / Initial weight of content (g)] x [Dilution Vol. (µL) / Volume aliquot in dilution (µL)] x [Number of copies / aliquot for qPCR reaction (µL)]

Relative Value (%) = (number of copies $_{\text{bacterial group}}$ / number of copies $_{\text{total bacteria}}$) x 100

In case the sum of group specific results was higher than total bacteria counts in a specific sample (as for stomach on days 0.5, 1, jejunum on days 0.25, 0.5, 1, and colon on days 0.5, 1, 3),

the calculation of the relative value was determined using the sum of all total copies of bacterial groups assuming a higher specificity of group specific analysis rather than total bacteria determination, justified by the fact that all summed values were in the range of standard error of numbers of copies $_{\text{total bacteria}}$. The fact that a rest error remained since not all bacteria were covered by the bacterial groups determined was acknowledged but dismissed due to detection method insufficiency.

3.3.4. Statistical Analysis

For statistical comparison of universal 16S rRNA gene sequences, ARB tree information was imported into Mothur software (version 1.12.0; http://www.mothur.org/; Schloss *et al.* 2009) and analyzed using the parsimony-based TreeClimber tool (Martin 2002; Schloss and Handelsman 2006) with 1,000 times randomized iteration. Pairwise comparisons were used to determine whether or not microbial communities have the same structure at a significance level of $P \leq 0.05$, trends were indicated for $0.10 \geq P > 0.05$. The TreeClimber parsimony tool determines whether or not two bacterial communities differ due to random variation or due to positive or negative selection pressure on bacterial lineages (Schloss and Handelsman 2006).

To analyze absolute qPCR data, number of copies per g of content results were normalized using $\log_{10}$ transformation, relative data was transformed using formula:

Normalized value (%) = [arc sin √ (rel. value (%) / 100)] x 100 (Garcia-Crespo *et al.* 2005)

Pearson correlation analysis was accomplished using correlation procedure of SAS (version 9.1.3; SAS Institute Inc. Cary, NC, USA). Preliminary statistical analysis was accomplished using Proc Mixed procedure of SAS using fixed effects gender, day of age and gastrointestinal location, the latter being analyzed as repeated measures within each animal over day of age. Litter was included in the mnodel as a randon effect. Since there were no significant effects or

interactions for gender and litter, these two factors were eliminated from the model. For repeated measure analysis, Proc Mixed options Simple, CS, CSH, TOEP, AR(1), ARH(1), UN and Ante(1) were compared. First Order Ante-Dependence (Ante(1)), allowing for unequal variances, correlations, covariance and spacing between repeats, was determined to be the best fit for analyzing repeated data. For all tests, significance was declared at $P \leq 0.05$, trends were indicated for $0.10 \geq P > 0.05$.

3.3.5. Nucleotide sequence accession numbers

Sequences have been deposited in the GenBank database under accession numbers HQ701141 to HQ701664.

3.4. Results

3.4.1. Sequence library composition

All strain specific information stated is based on RDP sequence alignments to type strains with similarities ≥ 97%. From 0.25 to 1 d of age a predominance of *Clostridium* spp. related (*C. perfringens* plus other *Clostridium* spp.) sequences (51.7%, 55.3%, 36.0%; combined for all 3 library locations on days 0.25, 0.5, 1, respectively) was observed in the GIT. *Escherichia* / *Shigella* spp. related sequences were second most abundant during this period representing 34.4, 14.8 and 3.8% of sequences combined for all locations on days 0.25, 0.5 and 1 respectively (Table 3.2). Representative species included *E. fergusonii* (n = 3/518), *S. dysenteriae* (n = 15/518) and *S. flexneri* (n = 13/518). *Clostridium perfringens* related sequences were the majority of sequences among *Clostridium* spp. between days 0.5 and 3 days of age (*C. perfringens* as percentage of *Clostridium* spp.: 91.1%, 89.4%, 74.8%, 66.1%; days 0.5, 1, 2, 3, respectively). Total *Clostridium* spp. and *C. perfringens* decline rapidly after 3 days of age (Table 3.2).

Table 3.2. Percent of total library sequences in GIT of neonatal suckling piglets, assigned to each genus.

		Day of Age							
Cluster (Genus level) [2]	N	0.25	0.5	1	2	3	5	10	20
Lactobacillus spp.	142	2.4		9.2	21.6	20.4	59.9	56.5	53.4
Streptococcus spp.	102	2.4	22.9	45.1	40.5	27.6	7.6	1.8	6.3
Clostridium perfringens [3]	82	23.2	50.4	32.2	8.9	10.9	1.6		
other *Clostridium* spp. [3]	47	28.5	4.9	3.8	3.0	5.6	7.8	8.6	12.5
Escherichia / *Shigella* spp.	38	34.4	14.8	3.8	3.0	1.3	1.6	5.1	
Moraxella spp.	21				4.2	10.5	7.6	4.5	2.9
Actinobacillus spp.	15		7.0	4.3	1.4	5.6	1.5	1.3	1.4
Sporobacter spp.	10	2.4						7.7	4.3
Veillonella spp.	8				7.0	3.8		1.5	
Peptostreptococcus spp.	7				1.5	8.7			
Bacteroides spp.	5				6.1				1.4
Phascolarctobacterium spp.	5						3.2	3.8	
Rothia spp.	5			1.3	1.4		1.5	1.5	1.4
Other spp. [4]	31	6.8			1.4	5.5	7.7	7.8	16.1
Total no. of sequence / day	518	52	68	71	62	73	65	67	65
Total (%) / day		100	100	100	100	100	100	100	100

[1] based on Greengenes NAST alignment (≥ 5 sequences). Libraries were constructed at each time point (0.25 - 20 d of age) for stomach, small and large intestine. Shadings show relative abundance of genus or subgroup for each day (solid black > 50.1%, solid dark grey 50.0 – 35.1%, dark grey pattern 35.0 – 15.1%, light grey pattern 15.0 – 5.1%, solid light grey 5.0 – 1.1%, white < 1.0%); [2] Cluster associated sequences > 80% similarity, except *Clostridium perfringens* subcluster ≥ 97 % similarity; [3] *Clostridium* genus cluster divided into subclusters '*Clostridium perfringens*' and 'other *Clostridium* spp.'; [4] Other species contain: *Dorea* spp. (n = 4), *Anaerotruncus*, *Neissera*, *Ruminococcus* spp. (n = 3 each), *Alistipes*, *Porphyromonas*, *Turicibacter* spp. (n = 2 each), *Aerosphera*, *Eubacterium*, *Fusobacterium*, *Helcoccus*, *Nosocomiicoccus*, *Parabacteroides*, *Prevotella*, *Spiroplasma*, *Weissella* spp. (n = 1 each).

Abundance of *Clostridium perfringens* sequences in each library also seemed to shift from predominance in the stomach to the intestine over the same period (data not shown). Interestingly, although our libraries were small, *C. perfringens* was not detected after 5 days of age (Table 3.2). Other *Clostridium* strains were mostly detected in the hindgut in low abundances (each n ≤ 6/518), namely *C. amygdalium, C. celatum, C. colicanis, C. disporicum, C. glycolicum, C. hathewayi, C. lituseburense, C. nexile, C. subterminale* and *C. symbiosum*.

Between days 1 and 3, *Streptococcus* spp. related sequences became transiently predominant (45.1%, 40.5%, 27.6% combined among locations on days 1, 2, 3, respectively, Table 3.2) and were detected in higher abundances in the SI versus stomach and LI (data not shown). *Streptococcus infantarius* (n = 57/518) was the major representative of the group, followed by *S. pyogenes* (n = 11/518). Both strains were not detected after 3 days of age. *Streptococcus suis* (n = 10/518) and *S. gallolyticus* (n = 3/518) were determined up to day 20 of age. Few sequences (each n ≤ 3/518) of *S. equinus, S. hyointestinalis, S. lutetiensis* and *S. porcinus* were detected.

Starting on day 5 of age, *Lactobacillus* spp. became the predominant sequences (59.9%, 56.5%, 53.0% combined among locations on days 5, 10, 20, respectively, Table 3.2) identified particularly in the stomach, although a shift to the LI was evident by 20 d of age (data not shown). *Lactobacillus mucosae* was the earliest identified *Lactobacillus* sp. on day 1 of age (n = 7/518), followed by *L. delbrueckii* (n = 37/518), *L. antri* (n = 4/518), *L. salivarius* (n = 13/518) on day 2 and *L. amylovorus* (n = 14/518), *L. johnsonii / L. gasseri* (n = 30/518), *L. vaginalis* (n = 3/518) between days 3 and 5, and *L. crispatus* (n = 5/518) after day 5 of age. *Lactobacillus delbrueckii* was the only species whose bacterial DNA was detected in the proximal GIT only. *Lactobacillus manihotivorans, L. pontis* and *L. zeae* were determined (each n = 1/518) in very low abundances.

A few *Bacteroides* and *Prevotella* spp. related sequences were detected as early as day 2 of age, representatives being *B. fragilis* (n = 1/518) and *B. vulgatus* (n = 1/518). Sequences related to other bacterial species were also detected in low abundances, namely *Moraxella canis* (n =

19/518), *Actinobacillus porcinus* (n = 7/518) and *A. minor* n = 6/518), *Veillonella caviae* (n= 7/518) and *V. criceti* (n = 1/518), *Peptostreptococcus stomatis* and *Rothia nasimurium* (each n = 5/518), *Dorea longicatena* and *Ruminococcus lactaris* (each n = 4/518), *Haemophilus parasuis* and *Neissera flavescens* (each n = 3/518), *Citrobacter koseri, Staphylococcus simulans* and *Turicibacter sanguinis* (each n = 2/518), as well as *Alistipes shahii, Anaerobacter polyendosporus, Eubacterium monoliforme, Fusobacterium necrogenes* and *Weissella confusa* (each n = 1/518). *Bifidobacterium* spp. related sequences were not recovered with universal 16S rRNA gene primers. However, when extracted DNA was pooled among all ages and location and amplified with *Bifidobacterium* spp. specific group primers, two *Bifidobacterium* spp. were recovered including *B. thermacidophilum* subsp. *porcinum* (n = 5/6 sequences) and *B. animalis* (n = 1/6).

3.4.2. Bacterial community structure

To emphasize the structural changes accompanying the sequence abundance changes described above, the TreeClimber parsimony tool was used to determine significant changes in bacterial community structure. Results were significant ($P \leq 0.05$) when ARB neighbor-joining trees were tested independently for either location or day of age (example: day 2 vs. day 20, see Figures 3.1 and 3.2). Since all structural changes were significant ($P \leq 0.05$) for both factors, the phylogenetic data was reanalyzed for each location per day to gain a better understanding of the structural changes observed during microbial succession. The resulting matrix is shown in Table 3.3. The bacterial community structure, exemplarily described for pig stomach on day 2, was significantly different ($P \leq 0.05$) due to positive or negative selection pressure on bacterial lineages from the structures of stomach and LI on day 0.25, all locations on day 0.5, stomach and SI on day 1, LI on day 5, stomach on day 5, and all locations starting day 10 of age.

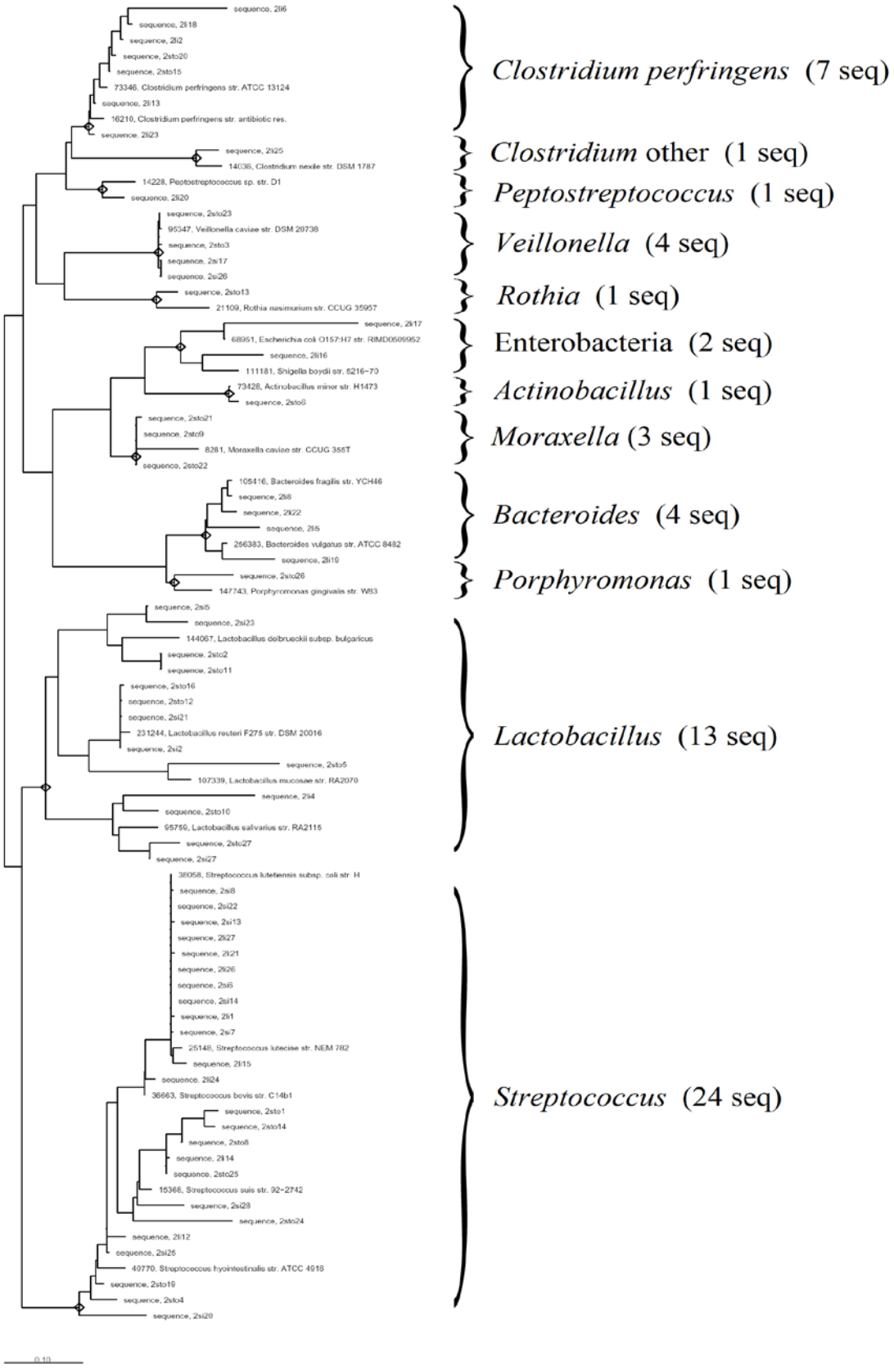

Figure 3.1. Neighbor-joining tree with bootstrap values removed, showing the phylogenetic relationships of sequences and reference strains recovered on day 2 of age. Square handles indicate grouping by genus, except for *Clostridium* (*C. perfringens* and *Clostridium* other). The bar indicates a calculated evolutionary distance of 10%; seq, number of sequences in group.

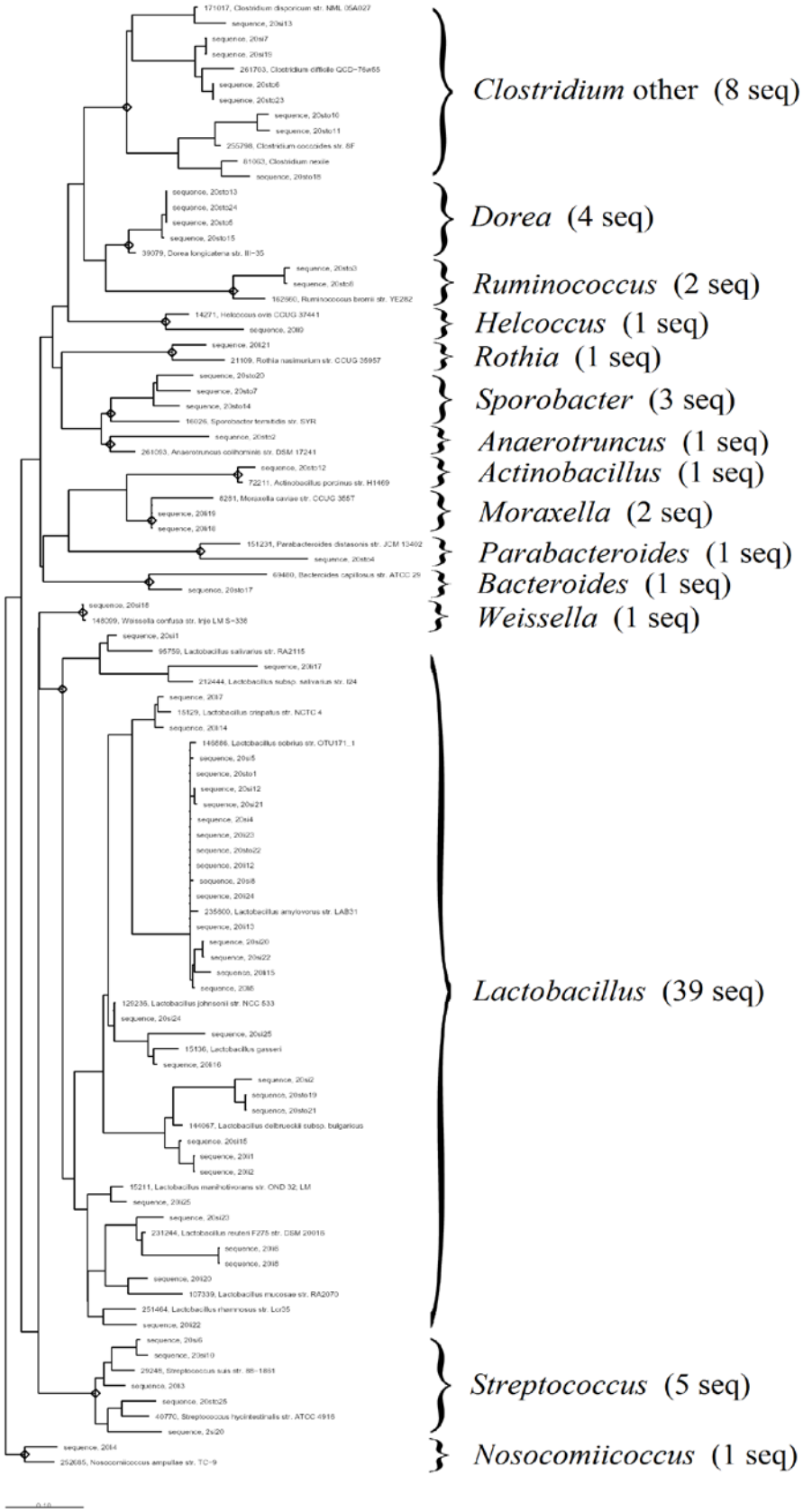

Figure 3.2. Neighbor-joining tree with bootstrap values removed, showing the phylogenetic relationships of sequences and reference strains recovered on day 20 of age. Square handles indicate grouping by genus, except for *Clostridium* (*C. perfringens* and *Clostridium* other). The bar indicates a calculated evolutionary distance of 10%; seq, number of sequences in group.

Table 3.3. Paired parsimony-based statistical analysis result matrix including sequence libraries prepared from all locations at all days of age investigated. [1]

		0.25			0.5			1			2			3			5			10			20	
		sto	si	li	sto	si	li	sto	si	li	sto	si	li	sto	si	li	sto	si	li	sto	si	li	sto	si
0.25	sto	x																						
	si	0.02	x																					
	li	<.02	>.10	x																				
0.5	sto	>.10	0.07	<.02	x																			
	si	>.10	>.10	0.02	>.10	x																		
	li	>.10	>.10	0.07	>.10	>.10	x																	
1	sto	>.10	<.02	<.02	>.10	>.10	>.10	x																
	si	<.02	<.02	<.02	0.02	>.10	0.05	>.10	x															
	li	0.07	>.10	<.02	>.10	>.10	>.10	0.02	>.10	x														
2	sto	<.02	>.10	<.02	<.02	<.02	<.02	<.02	<.02	>.10	x													
	si	<.02	0.02	<.02	<.02	<.02	<.02	0.07	>.10	>.10	>.10	x												
	li	0.02	>.10	<.02	>.10	>.10	>.10	>.10	>.10	>.10	>.10	0.05	x											
3	sto	<.02	0.02	<.02	<.02	<.02	<.02	0.05	<.02	0.05	>.10	>.10	>.10	x										
	si	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.02	0.05	>.10	>.10	0.07	>.10	x									
	li	<.02	>.10	<.02	<.02	0.05	>.10	>.10	<.02	0.05	0.05	<.02	>.10	>.10	0.02	x								
5	sto	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	>.10	<.02	0.02	x							
	si	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	>.10	<.02	<.02	>.10	0.05	>.02	>.10	x						
	li	<.02	0.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	>.10	<.02	0.02	>.10	0.05	>.10	>.10	>.10	x					
10	sto	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.02	<.02	<.02	>.10	<.02	<.02	>.10	>.10	>.10	x				
	si	<.02	<.02	<.02	<.02	<.02	0.02	<.02	<.02	<.02	<.02	<.02	<.02	>.10	<.02	0.05	>.10	<.02	0.05	>.10	x			
	li	<.02	>.10	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.05	<.02	<.02	x		
20	sto	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.02	0.02	<.02	<.02	<.02	0.02	>.10	<.02	<.02	0.10	x	
	si	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.05	<.02	>.10	<.02	<.02	>.10	>.10	>.10	>.10	>.10	<.02	<.02	x
	li	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	<.02	0.05	<.02	<.02	>.10	<.02	0.02	>.10	>.10	0.05	>.10	0.05	<.02	<.02	>.10

[1] sto, stomach; si, small intestine; li, large intestine; white, significant $P \leq 0.05$; grey, trend $0.05 < P \leq 0.10$; black, not significant $P > 0.10$.

Community structures were similar ($P > 0.05$) between stomach on day 2 and SI on day 0.25, SI on day 1, LI on day 2, Sto and SI on day 3, and SI and LI on day 5 indicating that the community structure differences derived from random variation (Table 3.3).

Over all, the bacterial community structure over the entire experiment organized in two major groups. The first encompassed samples taken from 0.25 to 2 days of age and the second included the samples from 3 to 20 days of age. In general the pairwise comparisons indicated similarity of community structure for each location between 0.25 and 2 days of age and for communities in each location between 3 and 20 days of age. However, pairwise comparisons outside these groups indicate the structures are different ($P \leq 0.05$). Thus, the preweaned phase could be distinguished between early (birth to 2 days of age) and late (days 3 to 20) preweaned phase.

3.4.3. Quantification of Selected Bacteria

Quantitative PCR (qPCR) was performed using unpooled digesta samples from stomach, jejunum (Jej) and colon (Co) on all sampling days for total bacteria counts, major bacterial groups (*Lactobacillus* spp. *Clostridium* cluster I (Ccl1) spp. *Streptococcus* spp. Enterobacteria) and selected minor groups of interest (*Bifidobacterium* spp. and *Bacteroides*/*Prevotella* spp.). Total bacteria counts increased with age from 9.27 $\log_{10}$ rRNA gene copies / g contents on day 0.25 of age to 11.15 $\log_{10}$ gene copies / g on day 20 of age and were higher ($P < 0.001$) in colon versus stomach. The succession pattern, based on quantification per gram of contents or as a percent of total eubacteria, was similar to that identified by analysis sequence data frequency in each library. *Clostridium* cluster I spp. (including *C. perfringens*) were predominant between days 0.25 and 0.5 with Enterobacteria spp. being the second most abundant on day 0.25. Between days 1 and 3, *Streptococcus* spp. became most prevalent, to be replaced on day 5 with *Lactobacillus* spp. the latter remaining most prevalent until day 20 of age. Compared to major groups, *Bifidobacterium* spp. and *Bacteroides*/*Prevotella* spp. groups were detected at lower

levels throughout the preweaning phase with higher variability compared to the most prevalent species (Figures 3.3 – 3.5). Significant effects of gastrointestinal location were observed for total bacteria, Clostridium Cluster I and Enterobacteria only, although the trend on all locations was increasing abundance form stomach to jejunum and jejunum to colon. A significant interaction between age and location was observed for enterobacteria abundance when expressed per gram contents such that log counts were highest in the stomach and jejunum at d 0.25 and highest in the colon at d 0.5 but abundance decreased steadily as animals increased in age (Figure 3.3-3.5).

Total bacteria, *Clostridium* cluster I and Enterobacteria showed significant location effects when expressed per gram contents reflecting increased abundance from proximal to distal GIT locations. Highest ($P < 0.01$) relative abundance (% of total eubacteria; Table 3.5) was observed for *Clostridium* cluster 1 and stomach and colon and for Enterobacteria in colon, whereas for Lactobacillus spp., highest relative abundance was observed in stomach and jejunum. Interestingly, *Streptococcus* and *Bacteroides*/*Prevotella* spp. abundance was not affected by location ($P > 0.10$; Table 3.4, 3.5).

3.5. Discussion

3.5.1. Sequence information

Fuller *et al.* (1978) determined that *Streptococcus* and *Lactobacillus* spp. including *S. bovis* (now *S. equinus*), *L. salivarius* and *L. delbrueckii* are resident in the stomach of the preweaned pig, and anticipated the presence of *Veillonella* and *Neissera* spp.. Using molecular methods, we were able to confirm these culture-based findings. Furthermore, Pederson and Tannock (1989) previously found *L. salivarius, L. crispatus* and other *Lactobacillus* spp. in the proximal gut using culture-based methods which is also similar to the findings in this study. Konstantinov *et al.* (2006) determined *Escherichia coli*, *Shigella flexneri*, *Lactobacillus* and *Streptococcus* spp. like *L. sobrius*, *L. reuteri* and *S. bovis* (now *S. equinus*), as well as *Clostridium* and *Neissera* spp. related species in the distal SI and LI of the preweaned pig, which was confirmed by findings in

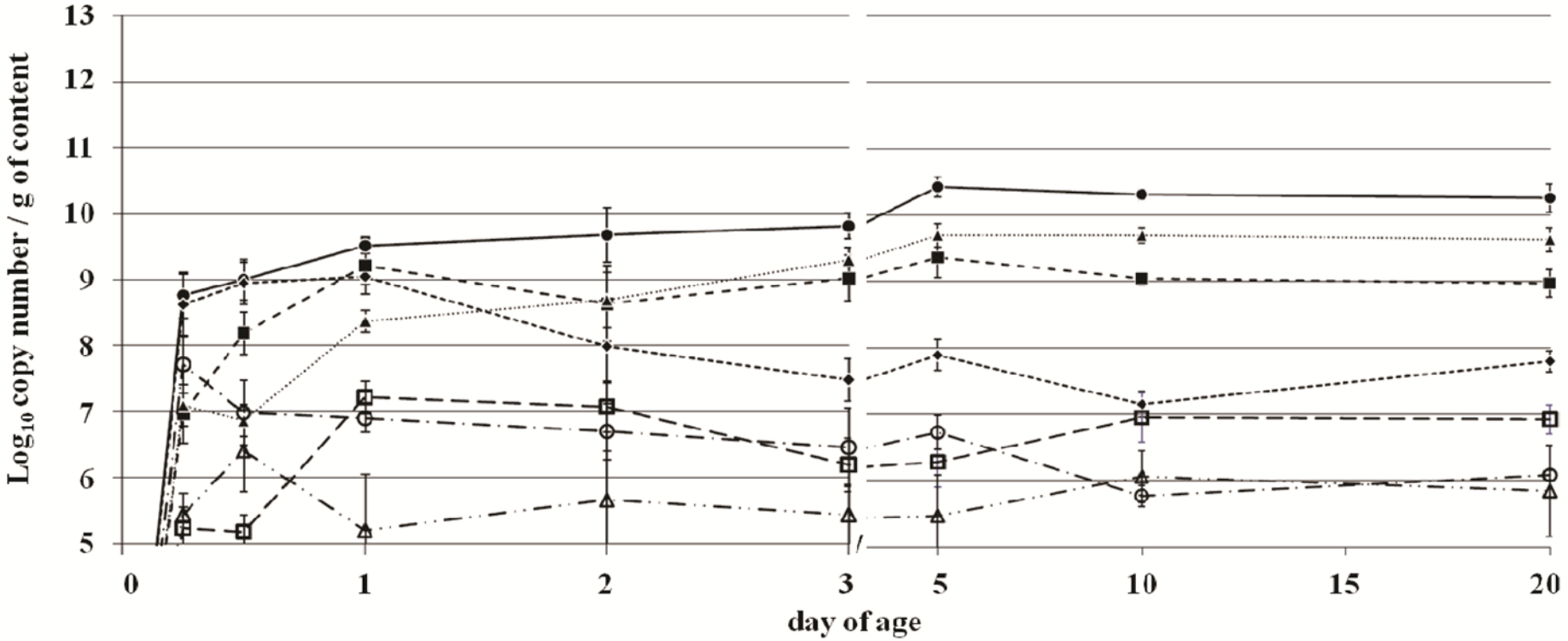

Figure 3.3. Mean abundance (log_{10} copies rRNA gene/g contents) of selected bacterial groups determined by qPCR for stomach; closed circles, total bacteria; closed diamonds, *Clostridium* cluster I spp.; closed squares, *Streptococcus* spp.; closed triangles, *Lactobacillus* spp.; open squares, *Bifidobacterium* spp.; open circles, Enterobacteria; open triangles, *Bacteroides*/*Prevotella* spp.; vertical bars indicate standard error.

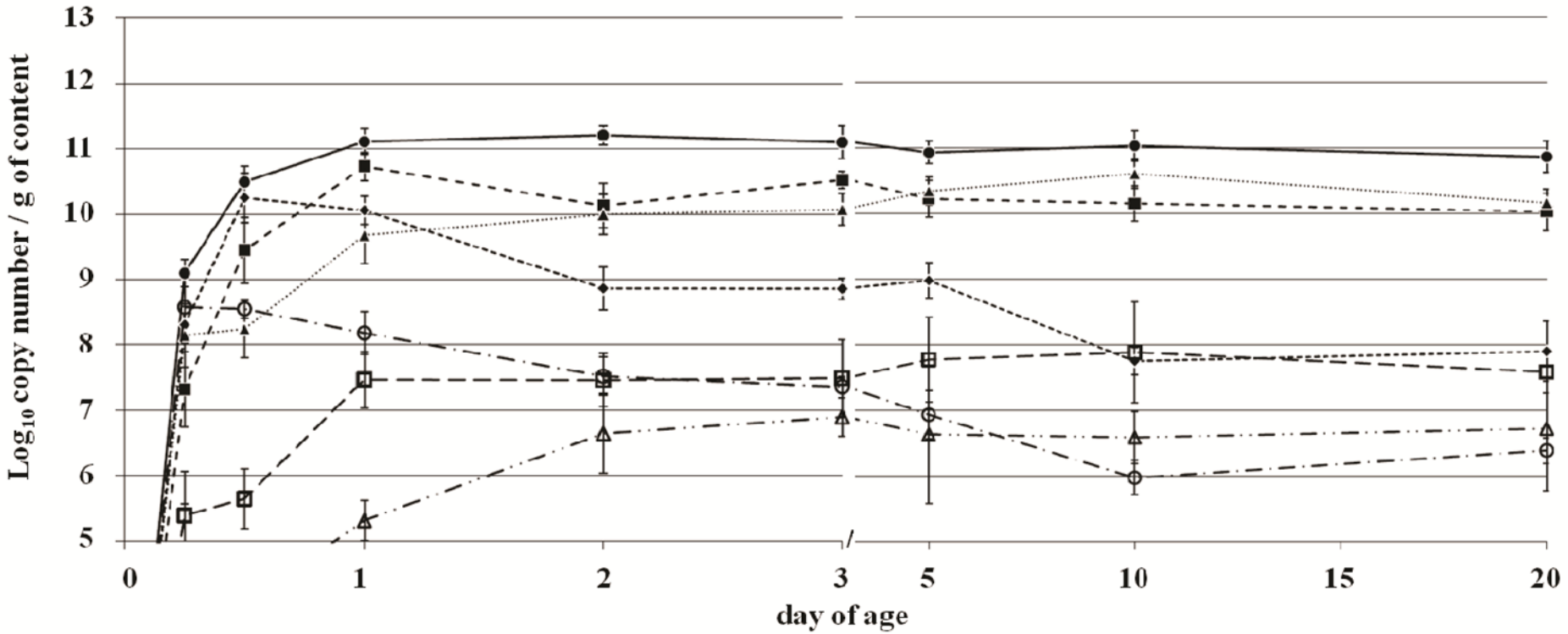

Figure 3.4. Mean abundance (log_{10} copies rRNA gene/g contents) of selected bacterial groups determined by qPCR for jejunum; closed circles, total bacteria; closed diamonds, *Clostridium* cluster I spp.; closed squares, *Streptococcus* spp.; closed triangles, *Lactobacillus* spp.; open squares, *Bifidobacterium* spp.; open circles, Enterobacteria; open triangles, *Bacteroides*/*Prevotella* spp.; vertical bars indicate standard error.

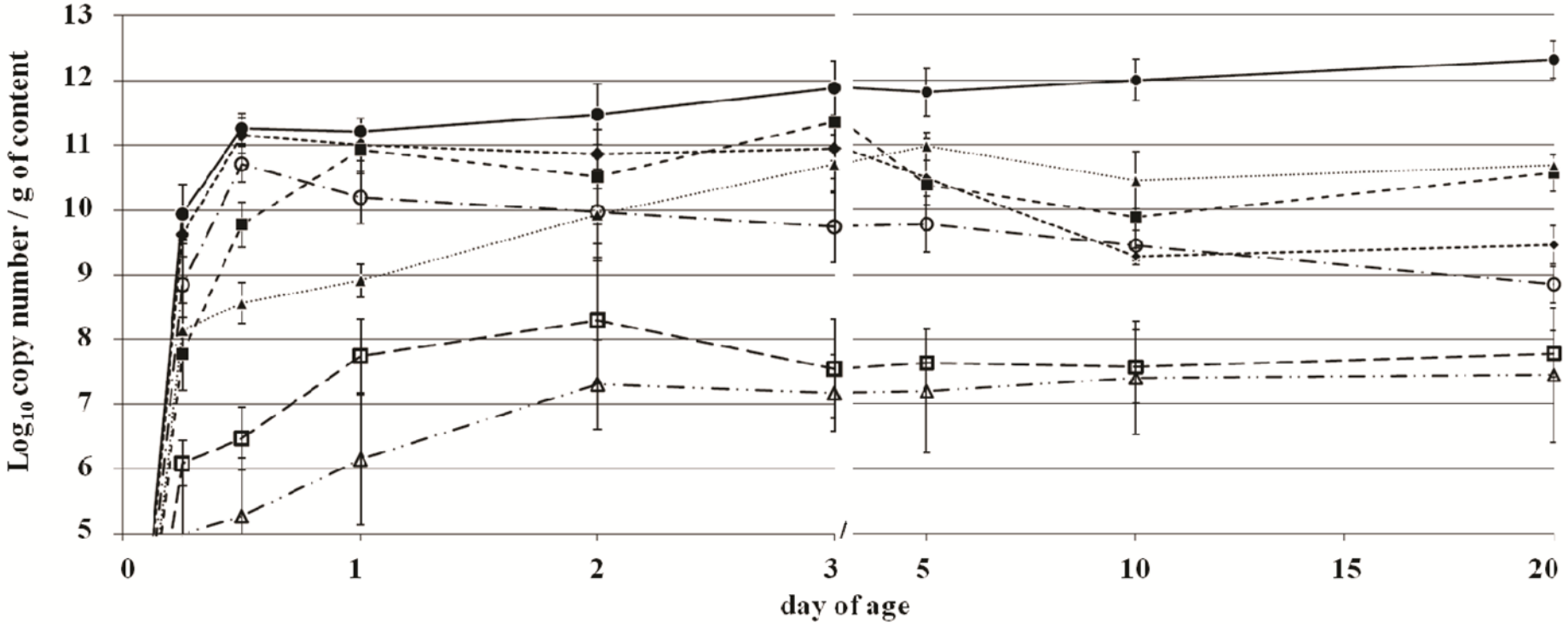

Figure 3.5. Mean abundance (log_{10} copies rRNA gene/g content) of selected bacterial groups determined by qPCR for colon; closed circles, total bacteria; closed diamonds, *Clostridium* cluster I spp.; closed squares, *Streptococcus* spp.; closed triangles, *Lactobacillus* spp.; open squares, *Bifidobacterium* spp.; open circles, Enterobacteria; open triangles, *Bacteroides*/*Prevotella* spp.; vertical bars indicate standard error.

Table 3.4. Mean abundance of major taxonomic groups by factors day of age and gastrointestinal location reported as $\log_{10}$ number of copies of 16S rRNA gene / g contents as determined by qPCR.

	Total [1]	**Ccl1** [2]	**Strepto** [3]	**Lacto** [4]	**Bifido** [5]	**Entero** [6]	**BaPr** [7]
Day of age							
0.25	9.27 [a]	8.86 [a]	7.35 [a]	7.79 [a]	5.57 [a]	8.38 [ab]	5.06
0.5	10.25 [b]	10.12 [b]	9.14 [b]	7.68 [a]	5.76 [a]	8.75 [b]	5.24
1	10.61 [bc]	10.04 [b]	10.29 [c]	8.89 [b]	7.48 [b]	8.43 [ab]	5.55
2	10.79 [bc]	9.24 [ab]	9.76 [bc]	9.54 [bc]	7.61 [b]	8.06 [ab]	6.54
3	10.93 [c]	9.10 [ab]	10.30 [c]	10.03 [c]	7.08 [b]	7.85 [ab]	6.50
5	11.06 [c]	9.12 [ab]	9.99 [bc]	10.34 [c]	7.23 [b]	7.81 [ab]	6.43
10	11.11 [c]	8.06 [a]	9.69 [bc]	10.25 [c]	7.47 [b]	7.06 [a]	6.68
20	11.15 [c]	8.38 [a]	9.85 [bc]	10.16 [c]	7.41 [b]	7.10 [a]	6.67
P-value	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*	*0.04*	*0.11*
SEM [8]	0.48	0.80	0.52	0.44	0.66	0.76	0.92
Location							
Stomach	9.72 [a]	8.12 [a]	8.68	8.67	6.38	6.67 [a]	5.69
Jejunum	10.73 [ab]	8.87 [a]	9.82	9.66	7.09	7.43 [b]	5.95
Colon	11.50 [b]	10.35 [b]	10.15	9.80	7.39	9.69 [c]	6.62
P-value	*<.001*	*<.001*	*0.13*	*0.20*	*0.40*	*<.001*	*0.33*
SEM [8]	0.26	0.30	1.10	0.86	0.91	0.62	0.97
Location x day interaction							
P-value	*0.28*	*0.11*	*0.93*	*0.77*	*0.97*	*0.02*	*0.85*

[a-c] Values with different letters in same column within day of age and location are different at $P \leq 0.05$; [1] Total, total bacteria; [2] Ccl1, *Clostridium* cluster I spp.; [3] Strepto, *Streptococcus* spp.; [4] Lacto, *Lactobacillus* spp.; [5] Bifido, *Bifidobacterium* spp.; [6] Entero, Enterobacteria; [7] BaPr, B*acteroides* / *Prevotella* spp.; [8] SEM, pooled standard error of the mean.

Table 3.5. Mean relative abundance of major taxonomic groups by factors day of age and gastrointestinal location reported as % of total bacteria as determined by qPCR. [1]

	Sum [2]	**Ccl1** [3]	**Strepto** [4]	**Lacto** [5]	**Bifido** [6]	**Entero** [7]	**BaPr** [8]
Day of age							
0.25	74.73	38.12 [d]	4.65 [b]	10.46 [bc]	0.206	19.83 [c]	1.461 [b]
0.5	85.75	58.40 [d]	13.36 [b]	0.95 [a]	0.005	11.74 [c]	1.296 [b]
1	77.99	25.32 [c]	38.28 [c]	6.62 [b]	0.259	6.60 [bc]	0.915 [ab]
2	50.16	10.94 [bc]	23.14 [c]	13.08 [c]	0.970	1.71 [a]	0.314 [a]
3	61.87	4.74 [b]	34.85 [c]	15.58 [c]	0.459	6.22 [b]	0.031 [a]
5	51.56	4.82 [b]	20.34 [c]	19.80 [c]	0.395	2.21 [ab]	1.167 [b]
10	32.66	0.16 [a]	7.89 [b]	23.49 [c]	0.892	0.18 [a]	0.043 [a]
20	30.39	0.40 [a]	9.28 [b]	19.59 [c]	0.154	0.03 [a]	0.933 [ab]
P-value		*<.001*	*<.001*	*<.001*	*0.41*	*0.05*	*<0.01*
SEM [9]		6.93	9.10	6.60	0.480	5.09	0.624
Location							
Stomach	58.27	18.56 [b]	18.88	17.51 [b]	0.567	1.82 [a]	0.934
Jejunum	57.54	9.93 [a]	22.90	18.56 [b]	0.518	3.90 [a]	0.696
Colon	58.61	25.10 [b]	15.15	5.04 [a]	0.168	12.47 [b]	0.680
P-value		*0.001*	*0.08*	*<.001*	*0.27*	*0.01*	*0.86*
SEM [9]		7.40	6.80	4.17	0.351	3.64	0.470
Location x day interaction							
P-value		*0.06*	*0.94*	*0.07*	*0.23*	*0.13*	*0.76*

[a-c] Values with different letters in same column within day of age and location are different at $P \leq 0.05$; [1] For samples within stomach on days 0.5, 1, jejunum on days 0.25, 0.5, 1, and colon on days 0.5, 1, 3, the sum of bacterial group counts were larger than determined total bacterial counts and were therefore used for calculation of percentages; [2] Calculated over relative abundance of bacterial groups; [3] Ccl1, *Clostridium* cluster I spp.; [4] Strepto, *Streptococcus* spp.; [5] Lacto, *Lactobacillus* spp.; [6] Bifido, *Bifidobacterium* spp.; [7] Entero, Enterobacteria; [8] BaPr, B*acteroides* / *Prevotella* spp.; [9] SEM, pooled standard error of the mean.

this study. Four sequences of *Clostridium difficile*, which can cause scours with typical razor sharp backs when overgrowth occurs (Songer and Taylor, 2006), were detected in stomach and jejunum in 20 day old pigs (Figure 3.4). Similar to other studies, small amounts of *Eubacterium* and *Fusobacterium* related species (Inoue *et al.* 2005), as well as *Bifidobacterium* spp. (Mikkelsen *et al.* 2003) were found, however the species detected in this study differed from previously described *Bifidobacterium* spp. in preweaned pigs.

3.5.2. Gastro-intestinal locations

Smith (1965) described differing microbial patterns between stomach and SI in numerous mammal species which emphasizes the importance of proximal GIT data in accessing succession patterns. Variation in proximal and distal GIT succession patterns was supported by several findings in this study, for example *L. delbrueckii* as major representative of *Lactobacillus* group was solely identified via sequences derived from Sto and SI (data not shown), and higher counts of *Bacteroides / Prevotella* spp. compared with *Bifidobacterium* spp. were observed on days 10, 20 in colon when comparing relative data only (Table 3.5). Also a significant location succession interaction for absolute Enterobacteria group data was observed, showing that Enterobacteria counts on day 0.5 were lower in stomach vs. jejunum which was reversed starting day 5 (Table 3.4). Total bacteria counts in stomach were unexpectedly high compared with previously reported total bacterial counts in stomach of pigs (Castillo *et al.* 2006a,b) and were presumably caused by multiple factors. Antibacterial components like IgA and lysozyme in maternal milk (Fan 2003) and, with the increasing age of the suckling piglet, low stomach pH reduce load of viable bacterial cells in the pig stomach (Dukes and Reece, 2004). Since nucleic acid degrading enzymes are not secreted in the stomach (Dukes and Reece, 2004), higher counts of bacterial

genetic material from dead bacteria were determined compared to viable cell counts, in addition to the already existing overestimation of bacteria counts due to 16S rRNA gene target bias (Fogel *et al.* 1999) and the difference to culture based methods since unculturable bacteria are quantified as well (Rigottier-Gois *et al.* 2003).

3.5.3. Structural changes of the microbial community

The TreeClimber parsimony tool in Mothur determines significant changes in bacterial community structure. Results were significant ($P \leq 0.05$) when ARB neighbor-joining trees were tested independently for either location or day of age. Since bootstrapped neighbor-joining trees are of high phylogenic quality (Schloss and Handelsman 2006), significant results were also obtained when tested within each day and location, even though the number of sequences per day and location were low. The overall most significant structural changes due to selective pressure on bacterial lineages occur between the grouped results from days 0.25 to 2 and day 3 to 20 and distinguish between early and late preweaned phase. It is interesting to speculate that this shift in the commensal bacteria population could be related to changes in maternal secretion. Colostrum, which is secreted from the mammary glands right after birth, is gradually replaced by milk until 48 h after parturition. Colostrum contains less water, fat and lactose than mature milk, but it contains about 3 times more protein. The protein in colostrum contains 91% whey protein compared to mature milk, which contains higher levels of casein (Darragh and Moughan 1998; Klobasa *et al.* 1987). In addition to the change of the nutritional composition of the bacterial environment in the GIT, the higher immune related factors like secretory IgA, specific pathogen antibodies, iron sequestering lactoferrin and lysozyme which are directly impacting gut microbiota composition (Thapa 2005), are much higher in colostrum compared to mature sow milk (Fan 2003). Those compounds in addition to host immune system stimulating prolin rich

polypeptide as well as epidermal, insulin like and transforming growth factors in colostrum (Thapa 2005, Fan 2003) are most important in regard to host epithelium maturation and prevention of microbial binding to IEC (Goldman and Goldblum 1989).

It should be acknowledged however, that the separation in microbial community structure observed between day 2 and 3 of age may be confounded by differences in litter of origin. Four of the six samples analyzed on each of the collection days between days 0.25 and 2 were from litters 1 and 2. Similary, four of the six samples analyzed for collection days between 3 and 21 were from litters 3 and 4. While litter effects on microbial composition have not been reported in the suckling pig, litter-of-origin effects have been observed in other species and may have played a role here.

3.5.4. Succession patterns

One of the earliest succession patterns determined by sampling feces of neonatal preweaned pigs describes a predominance of *E. coli* and *Streptococcus spp.* on day 0.25, followed by a predominance of *C. perfringens* being replaced by *Lactobacillus* spp. on day 2 of age (Ducluzeau 1985). These findings are somewhat similar to our study, with the exception that *Streptococcus* spp. and *C. perfringens* were interchanged, and species of Enterobacteria were not predominant at any point of time. The results reported in Ducluzeau (1985) do not allow for direct comparison to the findings presented here since findings were based on the observation of 10 animals only which were kept under experimental conditions (laboratory). Therefore this paper is the first to specify an intermediate predominance of *Streptococcus* spp. more accurately *S. infantarius*, in the microbial succession of the neonatal pig.

Seven years later, Swords *et al.* (1993) described a somewhat different microbial succession pattern in the neonatal pig colon. In their study, *Clostridium* spp. became predominant right after

birth with coliforms forming the second most prevalent group as was observed in the current study. Differing from results presented here, Swords *et al.* (1993) reported that *Clostridium* spp. stayed predominant during the whole preweaning phase. *Bacteroides* spp. were also detected early, but became 2nd most abundant before *Lactobacillus* spp. which differs from our findings of *Streptococcus* spp. being predominant, followed by *Lactobacillus* spp. (Table 3.2, Figure 3.5). The differences in results might be due to either or both, culture based bias (Gaskins 2001) or differing experimental conditions (open air barn) in Swords *et al.* (1993). Similar to findings of Konstantinov *et al.* (2006), higher small intestine enterobacteria counts were determined in two day old piglets compared with 19 day old suckling pigs, whereas *Lactobacillus* counts were higher in the older compared with younger pigs.

In our study, *Clostridium perfringens* was most abundant up to 12 hours of age, which indicated a predominance of a potentially pathogenic microorganism (Bourlioux *et al.* 2003) immediately after birth in healthy commercially reared neonatal pigs. This organism was rapidly displaced on day 1 of age by *Streptococcus* spp. and subsequently by *Lactobacillus* spp. on day 5 (Tables 3.2, 3.4, 3.5), which were categorized as a beneficial bacterial group (Bourlioux *et al.* 2003). Davis *et al.* (2007) showed that sow milk supplemented with beneficial bacteria like *Lactobacillus brevis* resulted in improved immune factors and increased body weight in weanling pigs. Beneficial microbe supplementation also impacted bacterial community structure in the pig at 20 kg body weight, even when *Lactobacillus brevis* supplementation was discontinued after weaning on day 20 of age. This suggests an effect of pre-weaning bacterial community changes on post weaning commensal bacteria structure and an impact of gut microbiota changes pre-weaning on post-weaning pig performance.

3.5.5. Individual animal variation

In regard to microbial variation between animals, a high individuality in colonic microbiota composition in suckling pigs before day 32 of age, as described in Thompson *et al.* (2008), was not seen in this study. Similar to their findings, the environment clearly overruled litter effects in regard to microbiota composition similarity with low standard errors in group specific analysis between non-related animals of the same age (Table 3.4, 3.5, Figures 3.3 – 3.5), proving the environment to have the strongest impact on the developing microbiota flora of the commercial neonatal pig.

3.6 Conclusions

A clear qualitative and quantitative microbial succession pattern along the entire GIT of the healthy standard commercial neonatal preweaned pig was established using molecular microbiological techniques. *Clostridium perfringens* and to a lesser extent species within the Enterobacteriaceae family were most abundant up to 12 hours of age, which indicates a predominance of harmful microorganisms immediately after birth. These organisms were initially displaced by 1 day of age with *Streptococcus* spp. mostly *Streptococcus infantarius* and subsequently *Lactobacillus* spp. which are categorized as beneficial bacterial group, from day 5 on. Furthermore, this succession pattern could be identified as two distinct but structurally similar phases separating the early preweaned phase, from birth up to day 2 of age, from the late preweaned phase, covering days 3 to 20 of age. The present data adds considerable sequence specific information regarding bacterial species colonizing the postnatal GIT, which is responsible for impacting the development of the postweaning pig. Low variability in individual animals kept in the same production facility was also shown here, indicating the importance of

production hygiene and proper pig management systems on herd health status. For future analysis, more information on regard to strain specific development, more locations along the GIT as well as comparisons of content and mucosa or epithelial cell attached microbiota might reveal further insights in with regard to microbial succession in the preweaned pig.

4.0 CHANGES IN MAJOR BACTERIAL TAXONOMIC GROUPS AND SELECTED MEMBER SPECIES WITH AGE AND GASTROINTESTINAL LOCATION IN COMMERCIALLY REARED PIGS

4.1. Abstract

The effects of maternal influence, piglet age and location within the gastro-intestinal tract on microbial succession of the neonatal commercial suckling pig were investigated using culture independent molecular methods of analysis. Collection of digesta at regular intervals between stomach and colon (stomach, duodenum, jejunum, ileum, cecum and colon) of 48 sow-reared piglets from 6 litters was done periodically over 20 days (d 0.25, 0.5, 1, 2, 3, 5, 10 and 20). The sampling scheme was controlled for effect of litter of origin and samples were analyzed by real-time quantitative polymerase chain reaction using 21 primers targeting individual species and total Eubacteria to permit absolute and relative quantification. Species composition within bacterial groups varied over time and within locations. Over all sections of the GIT, total bacterial counts were significantly lower ($P<0.05$) at day 2 versus day 20. The predominant differences were seen in *Lactobacillus*, *Clostridium* cluster IV and XIVa, and *Enterococcus* groups. All bacteria enumerated were significantly affected by GIT location except *Bifidobacterium*. Significant interactions were seen between age and location for *Clostridium* cluster IV and *Enterococcus*. Bacterial counts of major groups in mucosa were similar but reduced compared to bacterial counts in the digesta. However, this was not true for specific species within bacterial groups. Counts of bacterial species within the same genus changed differently in the upper gut compared with the hindgut, indicating that predictions made for the complete GIT by colonic or fecal sampling are questionable. No effect of litter was seen. The results of this trial indicate that the pig GIT and its microbial succession during development are

highly complex. However, a better understanding was gained by investigating longitudinal and radial changes within the pig GIT, as well as bacterial group specific changes temporally in the first 20 days of life for a conventionally raised pig.

4.2. Introduction

It is well known that first colonizing microbiota in the neonatal pig have an impact on both adult gut microbiota composition, and host-microbiota interaction, with possible long term implications on animal health and productivity (Tannock 2005; Mulder *et al.* 2009). Detailed knowledge of the microbial succession pattern in the commercial pig, including the upper GIT including digesta and mucosa-associated microbiota, remains limited despite numerous studies which have used both culture and culture-independent based approaches (Smith 1965; Fuller *et al.* 1978; Pederson and Tannock 1989; Ducluzeau 1985; Swords *et al.* 1993; Mikkelsen *et al.* 2003; Inoue *et al.* 2005; Konstantinov *et al.* 2005; Gancarčíková *et al.* 2008). Both culture (Swords *et al.* 1993) and culture-independent (Inoue *et al.* 2005; Thompson *et al.* 2008) methods have shown the importance of Clostridiaceae, Enterobacteriaceae and *Lactobacillus* families in the development of the pig through fecal analysis. Konstantinov *et al.* (2006), extended these observations and included culture-independent analysis of succession patterns in the pig ileum, but did not extend further proximally or to include mucosa-associated bacteria. The previous chapter (Chapter 3) further contributed to the knowledge of postnatal microbial succession pattern in the pigs, by examining proximal gastrointestinal colonization patterns during the early postnatal period at the genus level.

Maternal, spatial and temporal effects on microbial profile have been previously reported in the rodent (Deplancke *et al.* 2000; Gareau *et al.* 2006) and man (Bjorksten *et al.* 2001; Gueimonde *et al.* 2006) but have not yet been fully elucidated in the pig and therefore warrant

further examination. Based on previous studies in pigs and other mammals, it was hypothesized that early postnatal bacterial colonization patterns at the genus and species level will be affected by piglet age, longitudinal and radial gastrointestinal location and litter of origin. This experiment was therefore conducted to provide a more detailed profile of early postnatal bacterial succession patterns in the pig by sampling at multiple locations along the entire digestive tract in neonatal and 20 day old suckling pigs using molecular detection methods.

4.3. Materials and Methods

4.3.1. Animals and Sample Collection

Eight piglets (PIC Camborough®, Large White x British Landrace, PIC Canada Ltd. Winnipeg, MB, Canada) were each collected from 6 litters in a standard farrowing barn environment (Prairie Swine Inc., Saskatoon, SK, Canada). The experimental design was as described in Chapter 3. Piglets remained with the sow for the duration of the trial and were subject to routine processing at 1 day of age. To be able to determine a maternal influence on microbial succession the temporal sampling scheme was designed to control for litter of origin. Two piglets from each of litters 1 and 2 were killesd for each of the first 4 collection time points whereas 2 piglets from each of litters 3 and 4 were killed for each of the second 4 collection time points. One piglet from each of litters 5 and 6 were killed at each time point.

Animals were euthanized by emersion in CO_2 and exsanguination at 0.25, 1, 2, 3 and 20 d of age to permit collection of digesta from the stomach, duodenum [proximal one third of small intestine (SI)], jejunum [middle one third of SI], ileum [distal one third of SI], cecum and colon. Entire contents were removed from each segment and homogenized before sub-sampling for further analysis. For pigs on day 20 of age, an 8 cm segment of tissue from mid jejunum and colon were collected, snap frozen in liquid nitrogen and stored at -70 °C. Digesta samples were

immediately frozen on dry ice and stored at -70 °C until DNA extraction. Bacterial DNA was extracted as described by Dumonceaux *et al.* (2006). For mucosa samples, mid jejunum and colon segments were thawed, dissected longitudinally and after removing contents, the mucosa harvested by scrapping with a microscope slide. Mucosa samples were immediately processed for DNA extracted as described by Dumonceaux *et al.* (2006).The experimental protocol was approved by the University of Saskatchewan Animal Research Ethics Board (AUP Number 20070073) according to guidelines established by the Canadian Council in Animal Care (CCAC, 1993).

4.3.2. Bacterial Enumeration By Quantitative PCR

Quantitative real-time PCR (qPCR) was performed using previously described (Chapter 3.3) where samples were analyzed individually without pooling and a temporal sampling scheme was designed to control for litter of origin and maternal influence on succession patterns. Briefly, two piglets from each of litters 1 and 2 were killed for each of the first 4 collection time points (0.25, 0.5, 1, and 2d) whereas 2 piglets from each of litters 3 and 4 were killed for each of the remaining collection time points (3, 5, 10 and 20 d). Additionally, one piglet from each of litters 5 and 6 were killed at every time point. Analysis was also performed using newly developed primer sets targeting specific bacterial groups (Table 4.1). New primer sets were designed for species of interest based on previous research reported in chapter 3 using the NCBI primer search tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; Camacho *et al.* 2009). Due to the greater sequence diversity of *chaperonin*60 universal target (*cpn*60 UT) and its unique occurrence within the bacterial genome compared with 16S rRNA gene target (Hill *et al.* 2005), species specific qPCR primers were designed to target *cpn*60 UT. Primer specificity was confirmed by agarose gel (1.5%) electrophoresis of single-band PCR products prepared using a pool of genomic DNA from digesta samples as template. PCR products were gel purification

(QIAEX II Agarose gel DNA extraction kit, Qiagen), and sequenced (Sanger *et al.* 1977) at National Research Council, Plant Biotechnology Institute, (Saskatoon, Canada) using *cpn*60 UT qPCR forward and reverse primers.

Quantitative PCR was conducted using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad,). The following conditions applied: 1 x - 5 min, 35 x (melt curve from 65 °C to 95 °C, increment 0.5 °C - 5s (plate read). Amplification was performed using 13.0 µL reaction mixture containing template DNA and 6.5 µL iQ SYBR Green Supermix (BioRad). Amplification conditions were one cycle at 95 °C followed by 35 cycles of 95 °C for 30 s, primer specific annealing temperature (Table 4.1) for 40 s, and 72 °C for 40s. Standard curves were generated for each bacterial target using gel purified (QIAEX II, Qiagen), amplicons prepared by standard PCR using PCR conditions as described above for primer validation. Amplicons were quantified by $O.D._{260nm}$ and 6 serial 10 fold dilutions prepared starting with 5 pg / µl concentration. Standards were converted to copy numbers using the formula:

Number of copies = [DNA (pg) * 6.022 x 10^{23} (copies per mole)] / [fragment length (bp) * 1 x 10^{12} (pg / g) * 650 (g / mole of bp)]

All reactions included a no template control to verify lack of reagent contamination. All amplifications were followed by melt curve analysis (BioRad CFX manager software, version 1.6.541.1028) to ensure single product amplification. Duplicate threshold cycles (Ct) were averaged and mean values with greater than ± 0.50 standard deviations were reanalyzed.

Table 4.1. Primers used for quantitative PCR.

Name	Target	Sequence (5'-3') [1]	Ann. (°C)[2]	Amp. (bp)[3]	Origin
TotBac	16S rRNA gene V3 region, domain bacteria	CGGYCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	60	200	Lee *et al.* 1996
Ccl1	16S rRNA gene, genus *Clostridium* cluster I [4]	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	63	346	Song *et al.* 2004
Cperf	Alpha toxin gene, species *C. perfringens*	GAAGCTATGCACTATTTTGGAGAT ATACTGTTCTTTCCTTTCTTCTGC	55	120	Wilkie, *et al.* 2006
Ccl4	16S rRNA gene, genus *Clostridium* cluster IV [4]	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	50	239	Matsuki *et al.* 2006
Ccl14a	16S rRNA gene, genus *Clostridium* cluster XIVa [4]	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	50	440	Matsuki *et al.* 2002
Lacto	16S rRNA gene, genus *Lactobacillus*	GCAGCAGTAGGGAATCTTCCA GCATTYCACCGCTACACATG	60	349	Castillo *et al.* 2006a Walter *et al.* 2001
Lamyl	*cpn*60 universal target *L. amylovorus*/*sobrius*	CATCTAAAGAAGTTGGTGACT CAATGATTAACAAAGCCTTAC	60	291	Smith, 2006
Ldelb	*cpn*60 universal target species *L. delbrueckii*	CGAATCTGCGGTTTCAGTAGC GATGATACGGCAGCAACTTGG	58	100	Smith, 2006
Ljohn	*cpn*60 universal target species *L. johnsonii*	TACTATTGAAGAATCAAAGGG TCAGTAATCAAAATGTAAGGG	55	144	Smith, 2006
Lreut	16S rRNA gene, species *L. reuteri*	CAGACAATCTTTGATTGTTTAG GCTTGTTGGTTTGGGCTCTTC	60	304	Dommels *et al.* 2009
Strepto	16S rRNA gene, genus *Streptococcus*	AGAGTTTGATCCTGGCTCAG GTTAGCCGTCCCTTTCTGG	57	485	Nübel *et al.* 1996 Franks *et al.* 1998
Sequi	*cpn*60 universal target gene species *S. equinus*	CTATTGCTCAACCAGTTGCTAAC ACCTTCTACCACATCAAGTTCTG	53	176	Evaluated in current study
Sinfa	*cpn*60 universal target gene species *S. infantarius*	CGTGACTGCTGGAGCTAACC GATGAAACGGCTGCGACTTG	55	138	Evaluated in current study
Ssuis	*cpn*60 universal target gene species *S. suis*	GCTCTTCCTACACTTGTTCTC GGTTGGCAATGGCTTCAG	55	262	Evaluated in current study
Ecocc	16S rRNA gene, genus *Enterococcus*	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	60	144	Rinttilä *et al.* 2004
Entero	16S rRNA gene, family Enterobacteriaceae	ATGGCTGTCGTCAGCTCGT CCTCATTCTTTTGCAACCCACTC	60	385	Castillo *et al.* 2006a
Bifido	16S rRNA gene, genus *Bifidobacterium*	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	56	563	Matsuki *et al.* 2002
Banim	*cpn*60 universal target gene species *B. animalis*	CTCTCGTCAAGCAGCTTGTCG GGCCGTCTGGTCTTCCGCATT	68 [5]	239	Briggs, 2005
Btham	16S rRNA gene, *B. thermacidophilum*	TTGCTTGCGGGTGAGAGT CGCCAACAAGCTGATAGGAC	62	137	Mathys *et al.* 2008
BaPr	16S rRNA gene, Genera *Bacteroides & Prevotella*	GGTGTCGGCTTAAGTGCCAT CGGAYGTAAGGGCCGTGC	60	140	Rinttilä *et al.* 2004
Bfrag	*cpn*60 universal target gene species *B. fragilis*	CAATCGGTGTGGTGGAAGGTATG CGGTTCAAGGATAGGCAAGAAGTC	54	161	Evaluated in current study
Pbucc	*cpn*60 universal target gene species *P. buccalis*	GCTACCGTATCTGCTAACAAC TCCATCACACTCTCCATCTTG	56	206	Evaluated in current study

[1] top forward, bottom reverse; [2] Annealing temperature in °C; [3] Target amplicon size in base pairs; [4] by Collins *et al.* 2009; [5] two-step qPCR without 72 °C elongation.

Acceptable reaction efficiency (E) was set for the range 0.90 - 1.10, and standard curve R^2 values to ≥ 0.97. Results were expressed in $\log_{10}$ no. of gene copies per g of content or per g of mucosa.

4.3.3. Statistical Analysis

Statistical analysis was accomplished using Proc Mixed procedure of SAS using fixed effects gender, day of age and gastrointestinal location, the latter being analyzed as repeated measures within each animal over day of age. Also, random litter effects were determined by phase (day 0.25 – 2, day 3 – 20). Since there were no significant effects or interactions for gender and litter, these two factors were eliminated from the model. For repeated measure analysis, Proc Mixed options Simple, CS, CSH, TOEP, AR(1), ARH(1), UN and Ante(1) were compared. First Order Ante-Dependence (Ante(1)), allowing for unequal variances, correlations, covariance and spacing between repeats, was determined to be the best fit for analyzing repeated data. For all tests, significance was declared for $P \leq 0.05$, trends were indicated for $0.10 \geq P > 0.05$.

4.4. Results

4.4.1. Longitudinal Changes During Succession

Digesta samples collected from stomach, duodenum, jejunum, ileum, cecum and colon at 2 and 20 d of age were selected to represent the two phases of microbial succession (See chapter 3) and subject to detailed microbial profiling. Considered across all locations, total bacteria counts were 0.50 $\log_{10}$ copies/g content lower ($P < 0.05$) in 2 day old pigs compared with 20 day old pigs (Table 4.2; Figure 4.1). This response was also seen with reductions ($P < 0.05$) in the number of gene copies for *Clostridium* cluster IV, *Clostridium* cluster XIVa and *Enterococcus*, respectively (Table 4.2) with an associated location by day interaction ($P < 0.05$) in these same groups. The effect of gastrointestinal location on bacterial count was highly significant ($P \leq$

Table 4.2. Mean number of copies of selected major taxonomic groups in $\log_{10}$ copies 16S rRNA gene/g digesta collected at locations along the length of the GIT of suckling pigs at 2 and 20 days of age analyzed as factorial ANOVA with repeated location measures.

	TotBac [1]	BaPr [2]	Bifido [3]	Ccl1 [4]	Ccl4 [5]	Ccl14a [6]	Ecocc [7]	Entero [8]	Lacto [9]	Strepto [10]
Location										
Stomach	9.97 [b]	5.57 [a]	7.00 [ab]	7.89 [b]	6.05 [b]	5.52 [b]	6.83 [ab]	6.39 [b]	9.16 [ab]	8.80 [b]
Duodenum	8.86 [a]	5.97 [ab]	6.59 [a]	6.88 [a]	5.76 [ab]	3.85 [a]	7.28 [b]	5.89 [a]	8.61 [a]	7.73 [a]
Jejunum	11.04 [bc]	6.68 [ab]	7.52 [b]	8.38 [bc]	5.35 [a]	4.25 [b]	6.56 [a]	6.95 [b]	10.08 [b]	10.08 [c]
Ileum	11.10 [bc]	6.70 [ab]	7.35 [ab]	8.67 [b]	5.89 [ab]	4.27 [b]	8.50 [c]	7.46 [bc]	10.16[b]	9.99 [c]
Cecum	11.76 [c]	10.87 [c]	8.02 [b]	9.05 [b]	9.42 [c]	5.88 [b]	8.55 [c]	8.93 [cd]	9.97 [b]	9.83 [bc]
Colon	11.90 [c]	7.38 [bc]	8.03 [b]	10.16 [c]	8.76 [c]	6.74 [c]	7.66 [bc]	9.41 [d]	10.31 [b]	10.54 [c]
P-value	*<.001*	*<.001*	*<0.01*	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*
SEM [10]	0.67	0.81	0.54	0.63	0.51	0.59	0.64	0.93	0.51	0.62
Day of age										
2	10.52 [a]	8.05	8.35	9.14	6.25 [a]	4.94 [a]	7.16 [a]	8.27	9.95	10.07
20	11.02 [b]	8.51	8.09	9.04	7.49 [b]	5.55 [b]	8.13 [b]	8.22	10.51	10.11
P-value	*0.05*	*0.22*	*0.68*	*0.62*	*<.001*	*0.04*	*0.01*	*0.80*	*0.17*	*0.87*
SEM [11]	0.39	0.61	0.38	0.41	0.49	0.42	0.53	0.59	0.43	0.56
Location x Day of age interaction										
P-value	*0.93*	*0.91*	*0.72*	*0.82*	*0.02*	*0.08*	*<.001*	*0.16*	*0.45*	*0.21*

[a-d] values in same column and factor with differing superscripts are significantly different at $P \leq 0.05$; [1] TotBac, total bacteria; [2] BaPr, *Bacteroides / Prevotella*; [3] Bifido, *Bifidobacterium*; [4] Ccl1, *Clostridium* cluster I; [5] Ccl4, *Clostridium* cluster IV; [6] Ccl14a, *Clostridium* cluster XIVa; [7] Ecocc, *Enterococcus*; [8] Entero, Enterobacteria; [9] Lacto, *Lactobacillus*; [10] Strepto, *Streptococus*; [11] SEM, pooled standard error of the mean.

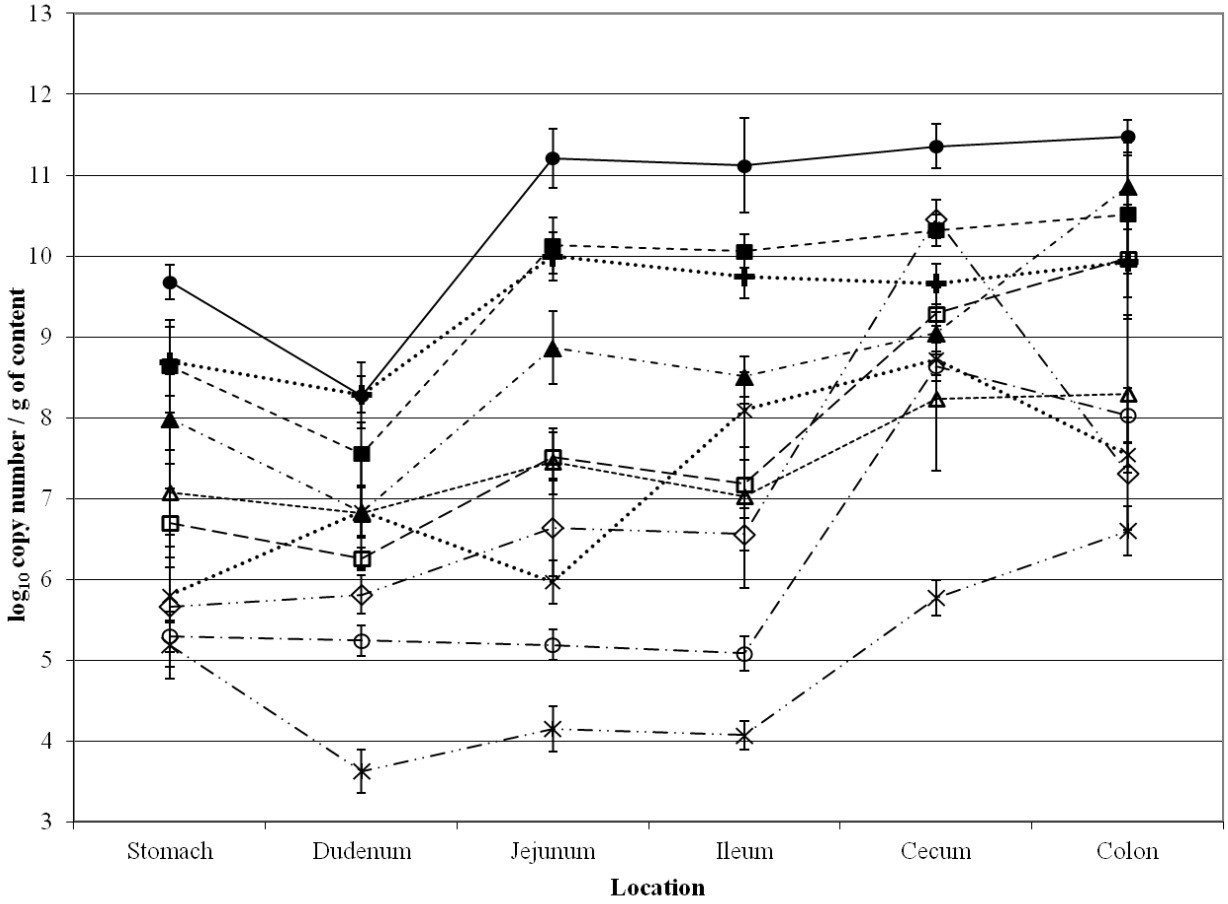

Figure 4.1. Mean number of copies of selected taxonomic groups (log_{10} copy number / g digesta) in digesta collected at locations along the length of the GIT of suckling pigs at 2 days of age; vertical error bars indicate standard error; filled circles straight line, total bacteria; filled triangles dash-dot line, *Clostridium* cluster I; filled square dashed line, *Streptococcus*; plus and dotted line, *Lactobacillus*; open squares dashed line, Enterobacteria; open triangles dotted line, *Bifidobacterium*; open diamonds dash dot line, *Bacteroides* / *Prevotella*; open circles dash-dot line, *Clostridium* cluster IV; star and dash-dot line, *Clostridium* cluster XIVa; x with dotted line, *Enterococcus*.

0.001) for all groups except for *Bifidobacterium* ($P < 0.05$). Total bacteria, *Clostridium* cluster I, *Clostridium* cluster XIVa, *Streptococcus*, *Lactobacillus*, Enterobacteria and *Bifidobacterium* were lowest in the duodenum whereas *Enterococcus* and *Clostridium* cluster IV were numerically lowest in jejunum. Generally, bacterial population density increased distally from duodenum with highest values observed in cecum and colon. *Clostridium* cluster I, *Clostridium* cluster XIVa, and Enterobacteria were significantly higher in the colon than elsewhere whereas *Bacteroides / Prevotella* and *Clostridium* cluster IV counts were highest in the cecum. Interestingly, *Bacteroides / Prevotella* declined significantly in mid-colon whereas *Clostridium* cluster IV did not. Significant interactions between age and location were observed for *Clostridium* cluster IV and *Enterococcus.* In the 2 day old pig, *Clostridium* cluster IV did not increase before the cecum (Figure 4.1), compared with 20 day old pigs where *Clostridium* cluster IV started increasing in the ileum (Figure 4.2).

4.4.2. Species Specific Enumeration

Analysis was performed for *B. fragilis* but was below detection level in individual animal samples and therefore data is not shown. *Bifidobacterium animalis* and *B. thermacidophilum* counts were relatively stable from stomach to colon and were not affected by location or age (Table 4.3) unlike the increasing trend for the *Bifidobacterium* group data for the same locations (Table 4.2). *Clostridium perfringens* colonization was lowest in ($P < 0.001$) in the jejunum compared to colon but was not affected by age (Table 4.3). *Prevotella buccalis* counts increased significantly over time from 3.59 to 6.07 $\log_{10}$ copy numbers per g of content without a location effect. The changes within the *Lactobacillus* group were more complex, mostly due to *L. amylovorus* and *L. reuteri* species specific changes (Figure 4.3). *Lactobacillus amylovorus* was lowest ($P < 0.05$) in jejunum versus the colon and tended to be higher ($P = 0.10$) in older pigs. *Lactobacillus reuteri* was highest ($P = 0.001$) in colon compared with other locations, and

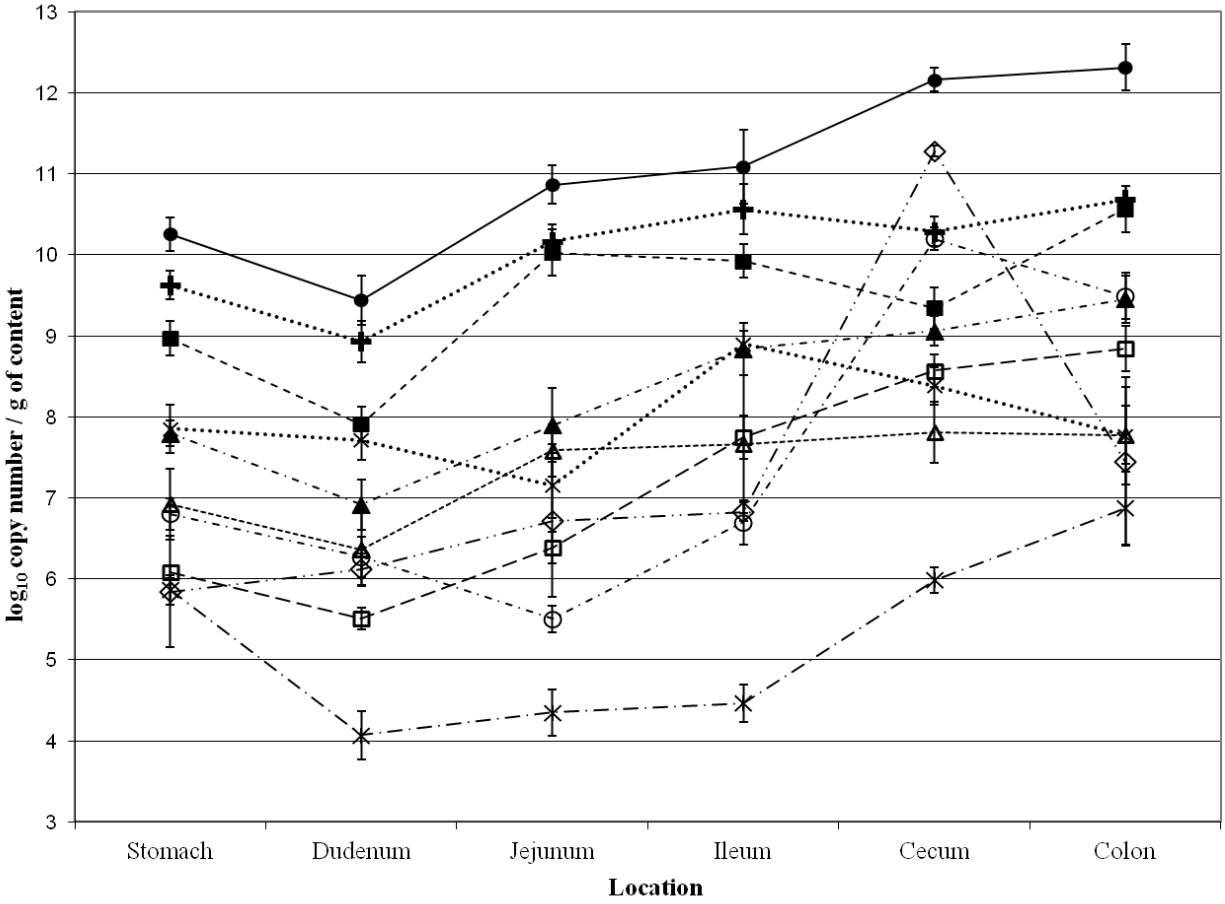

Figure 4.2. Mean number of copies of selected taxonomic groups (log_{10} copy number / g digesta) in digesta collected at locations along the length of the GIT of suckling pigs at 20 days of age; vertical error bars indicate standard error; filled circles straight line, total bacteria; filled triangles dash-dot line, *Clostridium* cluster I; filled square dashed line, *Streptococcus*; plus and dotted line, *Lactobacillus*; open squares dashed line, Enterobacteria; open triangles dotted line, *Bifidobacterium*; open diamonds dash dot line, *Bacteroides* / *Prevotella*; open circles dash-dot line, *Clostridium* cluster IV; star and dash-dot line, *Clostridium* cluster XIVa; x with dotted line, *Enterococcus*.

Table 4.3. Mean number of copies of selected bacterial strains (log_{10} copies 16S rRNA gene/g digesta) collected at locations along the length of the GIT of suckling pigs at varying time points for neonatal pigs and 20 days of age, depending on their group predominance or general structural difference as established in chapter 3. [1]

	Bifidobacterium [2]		Ccl1 [3]	*Lactobacillus* [4]				BaPr [5]	*Streptococcus* [6]		
	Banim	Btham	Cperf	Ldelb	Lamyl	Ljohn	Lreut	Pbucc	Sequi	Sinfa	Ssuis
Location											
Stomach	4.02	6.03	7.83 [ab]	6.17	7.88 [ab]	8.48	6.26 [a]	4.28	7.52	7.69	7.13
Jejunum	4.13	6.59	7.31 [a]	6.43	7.48 [a]	8.61	6.77 [a]	4.97	7.66	8.27	7.82
Colon	4.58	6.56	8.67 [b]	5.48	8.38 [b]	9.41	8.11 [b]	5.24	8.36	8.80	7.84
P-value	*0.39*	*0.56*	*<.01*	*0.70*	*0.05*	*0.27*	*0.001*	*0.51*	*0.25*	*0.09*	*0.31*
SEM [7]	0.29	0.58	0.53	0.39	0.42	0.46	0.45	0.48	0.34	0.44	0.28
Day of age											
≤ 3	4.20	6.91	8.39	5.70	7.45	9.00	8.48 [b]	3.59 [a]	8.65 [b]	9.10 [b]	7.02 [a]
20	4.28	5.87	7.84	6.35	8.66	8.67	6.05 [a]	6.07 [b]	7.05 [a]	7.41 [a]	8.17 [b]
P-value	*0.82*	*0.19*	*0.17*	*0.31*	*0.10*	*0.93*	*<0.01*	*<.001*	*0.03*	*<0.01*	*0.03*
SEM [7]	0.49	0.62	0.48	0.39	0.67	0.62	0.68	0.59	0.62	0.44	0.42
Location x Day of age interaction											
P-value	*0.40*	*0.31*	*0.27*	*0.26*	*0.02*	*0.06*	*<0.01*	*0.61*	*<0.01*	*0.01*	*0.43*

[a, b] values in same column and factor with differing superscripts are significantly different at $P \leq 0.05$; [1] sampling due to structural change on day 2 for *Bifidobacterium* and *Bacteroides/Prevotella* or first predominance of taxonomic group, day 0.25 for *Clostridium* cluster I, day 1 for *Streptococcus*, day 3 for *Lactobacillus*, as determined in chapter 3; [2] *Bifidobacterium* spp.: Banim, *B. animalis* and Btham, *B. thermacidophilum*; [3] Ccl1, *Clostridium* cluster I sp.: Cperf, *C. perfringens*; [4] *Lactobacillus* spp.: Ldelb, *L. delbrueckii*, Lamyl, *L. amylovorus*, Ljohn, *L. johnsonii* and Lreut, *L. reuteri*; [5] BaPr, *Bacteroides / Prevotella* sp.: Pbucc, *P. buccalis*; [6] *Streptococcus* spp.: Sequi, *S. equinus*, Sinfa, *S. infantarius* and Ssuis, *S. suis*; [7] SEM, pooled standard error of the mean.

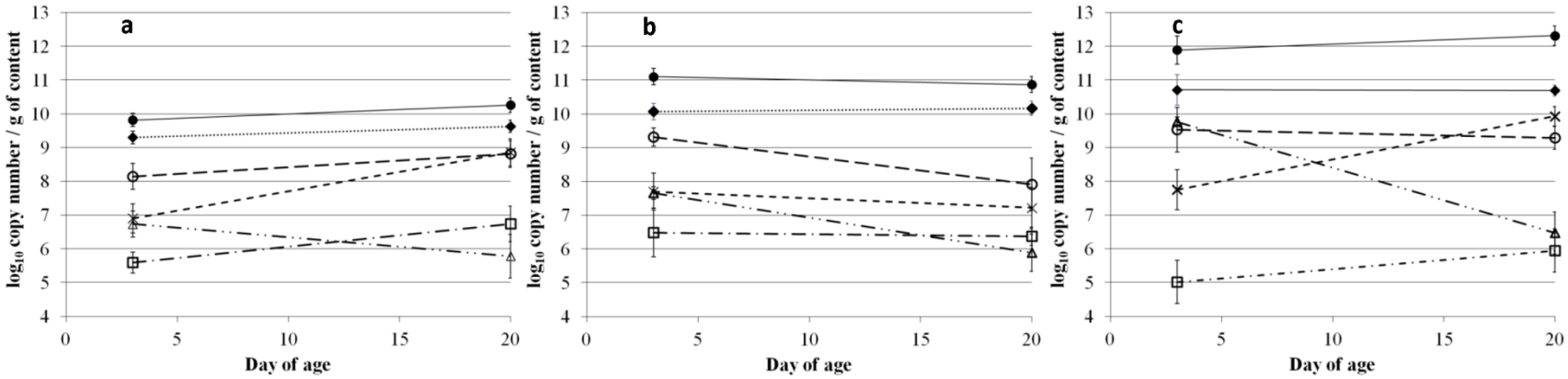

Figure 4.3. Mean number of copies of total bacteria and *Lactobacillus* species (log_{10} copies 16S rRNA gene/g digesta) collected at locations along the length of the GIT of suckling pigs (panel a, stomach; panel b, jejunum; panel c, colon) at 3 and 20 days of age; vertical error bars indicate standard error; filled circles straight line, total bacteria; filled diamonds dotted line, *Lactobacillus*; open circles dashed line, *L. johnsonii*; x with dashed line, *L. amylovorus*; open triangles dash-dot dot line, *L. reuteri*; open squares dash-dot line, *L. delbrueckii*.

declined ($P < 0.05$) with age. Both species had significant age by location interactions ($P < 0.05$) as shown in Figure 4.3. Although the initial density and rate of increase with age differed at each location, *Lactobacillus reuteri* decreased in all three locations starting at 2 days of age. *Lactobacillus amylovorus* increased in all three locations; most rapidly in the stomach. *Lactobacillus johnsonii* counts were increased with age in the stomach, decreased in the jejunum and stable in the colon. All species within the *Streptococcus* group were not affected by location but all were impacted by age and had *S. equinus* and *S. infantarius* showed significant ($P \leq 0.01$) location by age interaction (Table. 4.3; Figure 4.4). Counts for *S. equinus* and *S. infantarius* decreased from 2 to 20 days of age and were generally higher at both ages in distal compared with proximal locations. In contrast, *S. suis* counts were stable in stomach but increased with age in jejunum and colon (Figure 4.4).

4.4.3. Differences In Radial and Longitudinal Microbiota Group Counts In Pig Intestine At 20 Days Of Age.

The pattern of changes in bacterial count between digesta and mucosa in jejunum and colon was different for all bacteria resulting in a significant interaction for quantified groups except *Bacteroides/Prevotella*. Total bacteria counts per gram of jejunal or and colonic mucosa were lower ($P \leq 0.01$) compared with the digesta from the same location. The difference was almost 2.66 $\log_{10}$ 16s RNA copies in colon compared with a 1.29 $\log_{10}$ reduction in jejunum (Table 4.4). In the jejunum, most bacterial groups were either numerically (*Clostridium* cluster I, *Streptococcus*, Enterobacteria, *Enterococcus*) or significantly (*Lactobacillus*, *Clostridium* cluster IV,) lower in the mucosa compared with the digesta. However, *Bacteroides / Prevotella* ($P = 0.08$), *Bifidobacterium* ($P < 0.05$,) and *Clostridium* cluster XIVa ($P < 0.001$) were higher in mucosa compared to digesta. In colon with the exception of *Bacteroides /Prevotella*, all bacterial

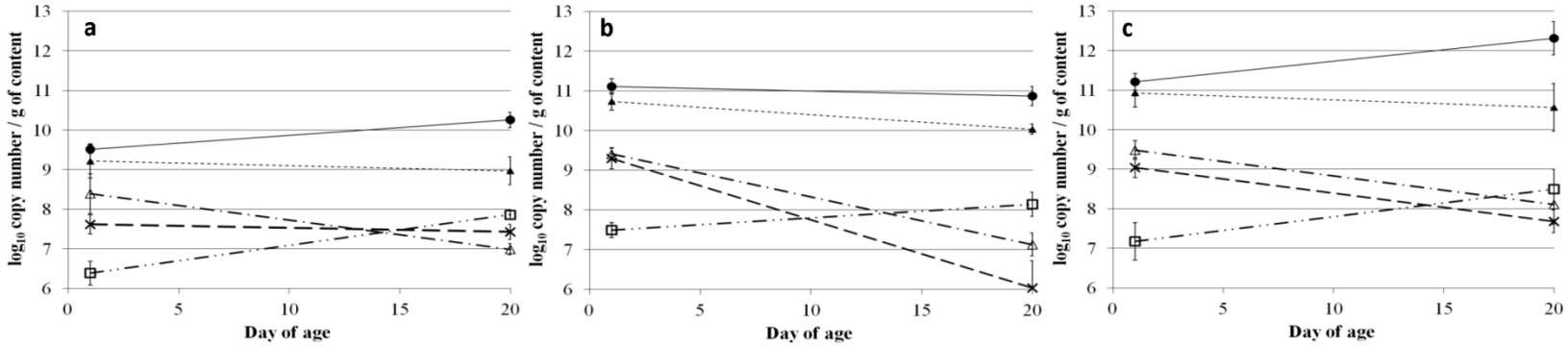

Figure 4.4. Mean number of copies of total bacteria and *Streptococcus* species (log_{10} copies 16S rRNA gene/g digesta) collected along the length of the GIT of suckling pigs (panel a, stomach; panel b, jejunum; panel c, colon) at 1 and 20 days of age; Vertical error bars indicate standard error; filled circles straight line, total bacteria; filled triangle dashed line, *Streptococcus*; open triangle with dash-dot line, *S. infantarius*; x with dashed line, *S. equinus*; open squares dash-dot dot line, *S. suis*.

Table 4.4. Mean number of copies of selected bacterial strains (log_{10} copy number of 16S rRNA gene/g digesta or mucosa) collected at locations along the length of the GIT of suckling pigs at 20 days of age.

	TotBac [1]	BaPr [2]	Bifido [3]	Ccl1 [4]	Ccl4 [5]	Ccl14a [6]	Ecocc [7]	Entero [8]	Lacto [9]	Strepto [10]
Jejunum										
Digesta	10.87 [b]	6.72	7.58 [a]	7.90	5.50 [b]	4.35 [a]	7.16	6.39	10.16 [b]	10.03
Mucosa	9.58 [a]	7.08	8.63 [b]	7.12	4.59 [a]	5.51 [b]	6.63	6.07	9.01 [a]	9.47
P-value	*0.01*	*0.28*	*0.02*	*0.16*	*0.02*	*0.01*	*0.10*	*0.33*	*0.001*	*0.33*
SEM [11]	0.38	0.42	0.63	0.79	0.60	0.42	0.72	0.51	0.43	0.45
Colon										
Digesta	12.31 [b]	7.45	7.77 [b]	9.45 [b]	9.49 [b]	6.87 [b]	7.65 [b]	8.84 [b]	10.69 [b]	10.57 [b]
Mucosa	9.65 [a]	6.89	5.17 [a]	8.35 [a]	8.35 [a]	5.54 [a]	3.84 [a]	6.47 [a]	8.23 [a]	8.01 [a]
P-value	*<.001*	*0.85*	*<.001*	*<0.01*	*0.02*	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*
SEM [11]	0.47	0.47	0.50	0.56	0.40	0.34	0.74	0.69	0.52	0.48
Interaction										
P-value	*<.001*	*0.21*	*0.01*	*<.001*	*<.001*	*0.01*	*<0.01*	*<.001*	*<.001*	*<0.01*

[a, b] values in same column and factor with differing superscripts are significantly different at $P \leq 0.05$; [1] TotBac, total bacteria; BaPr, *Bacteroides / Prevotella*; Bifido, *Bifidobacterium*; Ccl1, *Clostridium* cluster 1; Ccl4, *Clostridium* cluster IV; Ccl14a, *Clostridium* cluster XIVa; Ecocc, *Enterococcus*; Entero, Enterobacteria; Lacto, *Lactobacillus*; Strepto, *Streptococus.*

groups were significantly reduced ($P < 0.05$) in mucosa compared to digesta. Of all groups, *Enterococcus* spp. demonstrated the most dramatic decline (3.81 $\log_{10}$ 16S rRNA gene copies per gram) in population density between colon digesta and mucosa (Figure 4.5).

4.5. Discussion

4.5.1. Gut Microbiota Differences in over various Locations and Age

In general, research comparing microbial succession and locations is limited, especially in preweaned pigs since most work was done in colon or via fecal matter and traditional microbial counts (Swords, *et al.* 1993). Castillo *et al.* (2006a) examined bacterial populations in several intestinal locations in post-weaned pigs at 6 weeks of age. Generally, population densities reported here are in agreement with this work with the exception of the stomach. Interestingly, the counts presented by Castillo *et al.* (2006a) for stomach digesta were about 1 log lower than determined in this study. This might be due to the lack of a fully established stomach acid barrier of pigs both pre- and early post weaning (Cranwell, 1995; Ravidran and Kornegay, 1993) and/or the fermentative characteristics of milk compared with solid diets (Barrow *et al.* 1977). Castillo *et al.* (2006b) reported that molecular enumeration of bacteria yielded counts 2-3 $\log_{10}$, higher than compared with culture based methods. Our previous comparison of culture and molecular based enumeration also noted a similar discrepancy in counts between methods (Chapter 3). Methodological differences thus are likely to explain the very high microbial counts reported here compared to results via culture based analysis methods (Swords *et al*. 1993).

Investigation of changes during succession over all GIT locations sampled (Table 4.2, Figure 4.1a and b) indicated that microbial changes along the GIT of the suckling pre-weaned pig at 2

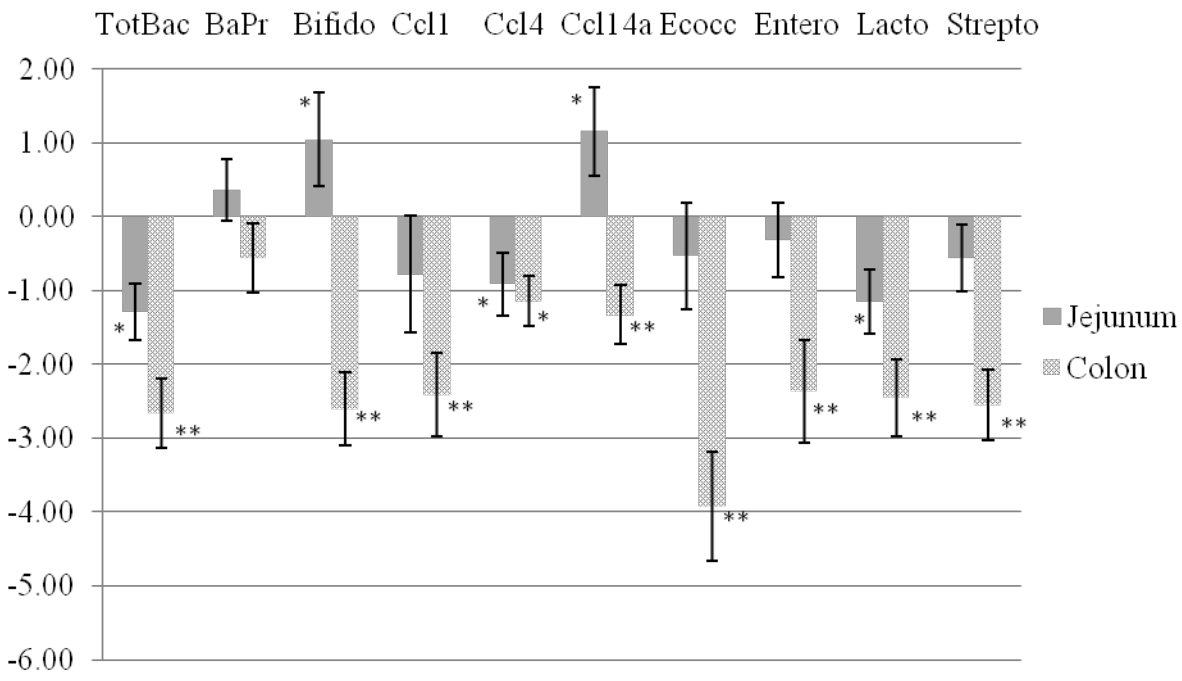

Figure 4.5. Difference in bacterial density (log_{10} copies 16S rRNA gene/g mucosa minus digesta) in jejunum or colon of suckling pigs at 20 days of age. Error bars indicate accumulated standard error of both measurements performed; TotBac, total bacteria; BaPr, *Bacteroides* / *Prevotella*, Bifido, *Bifidobacterium* group; Ccl1, *Clostridium* cluster I; Ccl4, *Clostridium* cluster 4; Ccl14a, *Clostridium* cluster XIVa; Ecocc, *Enterococcus* group; Entero, Enterobacteria group; Lacto, *Lactobacillus* group; Strepto, *Streptococcus* group.

and 20 days of age were quite similar. Similar to findings of Konstantinov *et al.* (2006), *Lactobacillus* counts increased in ileum over time, even though the levels were generally lower in their study. Bacterial counts in the duodenum were affected by bile salts and digestive enzymes secreted by pancreas and gall bladder (Dukes and Reece, 2004), which may have contributed to the consistently lower counts of major bacterial groups in this location. Predominance of *Bacteroides / Prevotella* group associated bacteria in the cecum on both days was a significant finding. Members of these genera mostly ferment carbohydrates to the volatile fatty acids (VFA) acetate and succinate, as well as lactate, iso-butyrate and iso-valerate (Holt *et al.* 2000). The predominance of these genera in the cecum is likely to be beneficial for the host, possibly due to absorption of VFA produced by *Bacteroides / Prevotella*. The assumption of *Bacteroides / Prevotella* being beneficial for the host in some way was also supported by the finding that *Bacteroides / Prevotella* bacteria also colonized the mucosa (Table 4.4, Figure 4.4). *Bacteroides / Prevotella* species as well as enterobacteria, which were found in reduced counts in the mucosa only, are gram negative bacteria and thus carry abundant lipopolysaccharides on their outer cell wall. Lipopolysaccharide molecules in close proximity to the epithelial cells are sensed by the host via TLR4, and subsequently trigger an immune response unless this process is down regulated in another place in the signaling cascade (Hoshiro *et al.* 1999). Location changes also revealed that in the jejunum, *Bifidobacterium* and *Clostridium* cluster XIVa were also tolerated in close proximity to the intestinal epithelium. *Bifidobacterium* species are generally believed to be beneficial for the host. Due to the findings that *Bifidobacterium* colonizes the mucosa in the jejunum but only to a very low amount in the colon, it is likely that the beneficial effect of probiotic *Bifidobacterium* is to be found as an interaction with the host in the small intestine. This is an interesting finding since it is well known that jejunum mucosa is rather aerobic due to

villus oxygen supply (Dukes and Reece, 2004) and thinnest mucus layer (Atuma *et al.* 2001). Nevertheless, it was determined that intestinal *Bifidobacterium* strains have the capability to adhere to epithelial surfaces (Pérez *et al.* 1997), even though *Bifidobacterium* is strictly anaerobic (Bergey and Holt, 1994). Regarding other mucosa colonizing species, it was determined that *Clostridium* cluster XIVa and *Bacteroides / Prevotella* were previously associated with cellulolytic activity in the ileum of pigs fed carbohydrates resilient to the digestive enzymes of the host (Metzler *et al.* 2010). The study indicated a beneficial nature of *Clostridium* cluster XIVa and *Bacteroides / Prevotella* species, increasing nutrient availability of the host by breaking down cellulolytic material. Similar to findings here, Wang *et al.* (2003) determined predominantly *Bacteroides / Prevotella* and *Clostridium* species in ileal mucosa in humans. Tolerating these bacterial groups in the mucosa being in close proximity to epithelial cells might be an inherited trait in the pig GIT. In addition to that, *Bacteroides* and *Prevotella* species have been shown to be capable to adhere intestinal surfaces (Kishore and Sotirios, 2009; Pumbwe *et al*. 2007), which might explain their increased numbers in jejunum but not in colon mucosa (Table 4.4, Figure 4.4). Another example for possible inheritance or niche occupation during maturation was shown in the *Clostridium* cluster IV group. *Clostridium* cluster IV species were associated with soluble fiber digestion in the stomach and hindgut of the pig (Castillo *et al.* 2007). Although there was no fiber from plant material present in the current study, suckling piglets were harboring increasing amounts of *Clostridium* cluster IV over time. The combined findings indicate incapacity of *Clostridium* cluster IV bacteria to colonize the small intestine, possibly due to an increased passage rate of digesta in the small intestine. Interestingly, the distinct pattern in this study was consistent with findings in Castillo *et al.* (2007), determining intermediate amounts of *Clostridium* cluster IV in stomach, lowest amounts in jejunum and

highest amounts in the hindgut of pigs via fluorescent *in situ* hybridization (FISH) technique. The level of total bacteria were lower in Castillo *et al.* (2007) probably due to the fact that FISH analysis requires very different sample preparation procedures compared with qPCR in this study.

4.5.2. Species Specific Determination within Bacterial Groups

Strain specific analysis (Table 4.3, Figures 4.) revealed that *C. perfringens* was detected lower than *Clostridium* cluster I, which indicated that there were no major changes within the *Clostridium* cluster I composition. *Clostridium perfringens* was not able to colonize in the small intestine over time, which possibly relates to *C. perfringens* as a toxin producer being actively repelled by the host in the small intestine. Species within *Bifidobacterium* genus showed no variation in composition due to location and age. *Prevotella buccalis* counts increased with age in all locations over proportional to *Bacteroides / Prevotella* development, indicating changes within the *Bacteroides / Prevotella* composition over time. Most remarkable were the changes within the *Lactobacillus* and *Streptococcus* group. *Streptococcus* species were over all unaffected by GIT location, but were significantly altered by age and as a result of an age x location interaction, indicating a high variability within the *Streptococcus* group. *Lactobacillus* species were affected by location and age, as well as their interaction, which indicated an even higher variability within the *Lactobacillus* group compared with the *Streptococcus* group. Konstantinov *et al.* (2006) compared *L. reuteri* and *L. amylovorus* over time, the latter described as *L. sobrius* there and renamed to *L. amylovorus* by Jakava-Viljanen *et al*. (2008). *Lactobacillus reuteri* and *L. amylovorus* were determined around 7.5 $\log_{10}$ copy numbers / g of ileal content on day 2 of age, similar to findings here for both species in pig jejunal contents on day 3 of age. However, the ileal *L. reuteri* counts remained constant and *L. amylovorus* counts increased on

day 19 in Konstantinov *et al.* (2006), whereas in this study, *L. reuteri* counts decreased and *L. amylovorus* counts remained constant in jejunal contents of 20 day old piglets. While Konstantinov *et al.* (2006) measured *Lactobacillus* spp. in the ileum and measures in this study were determined in the jejunum, no other published research is available for *Lactobacillus* spp. comparison in the suckling pig gastro-intestine tract according to the author's knowledge.

4.6. Conclusion

The present study provides a detailed comparison of microbial communities in intestinal contents and mucosal along the entire GIT and between 2 and 20 days of age. The age-related increase of lactic acid bacteria described here were similar to findings of Konstantinov *et al.* (2006), and resembled in general the bacterial patterns shown by culture based methods by Swords *et al.* (1993). The findings indicated that microbial succession in the pig is highly complex. A better understanding was gained by investigating longitudinal and radial changes within the pig GIT, as well as determining a better insight in bacterial group specific changes. Species compositions within bacterial groups varied over time and within locations, but animal-to-animal variation remained surprisingly small. Bacterial counts of major groups in digesta were similar but reduced compared to bacterial counts in the mucosa. However, this was not true for specific species within bacterial groups. Representatives of both *Bifidobacterium* and *Bacteroides / Prevotella* have been shown to be capable to adhere to epithelial surfaces (Kishore and Sotirios, 2009; Pérez *et al.* 1997; Pumbwe *et al.* 2007) and seem to be more numerous in jejunal mucosa potentially due to the mucus layer being thinnest compared with the remaining digestive tract (Atuma *et al.* 2001). Upper gut bacterial composition in bacterial groups changed inconsistently with changes in the hindgut, indicating that predictions made for the complete GIT by colonic or fecal sampling are questionable.

5.0 A SNATCH-FARROW, ISOLATOR REARING APPROACH IN THE PIG AS A MODEL TO CONTROL EARLY POSTNATAL MICROBIAL COLONIZATION

5.1. Abstract

To test the hypothesis that different early postnatal bacterial colonization patterns modify colonization profile after weaning a method of reliably controlling postnatal colonization was investigated. Newborn pigs (n = 15) were collected immediately from the vaginal canal from one of 3 sows and placed in one of six sterile HEPA-filtered containment isolators such that ½ the pigs from each sow were housed in an individual isolator. One half of piglets from each sow were exposed to sow feces within 24 h of birth while remaining piglets were not inoculated. All pigs were inoculated with sow feces at 4 d of age and subsequently removed from isolators, reared with a milk substitute until 20 d of age, weaned to a cereal-based diet and euthanized at 28 d of age. Terminal Restriction fragment polymorphism (TRFLP) and qPCR were employed to confirm treatment effects on early postnatal and postweaning intestinal microbial profile. Inoculation treatment significantly ($P \leq 0.01$) affected *Lactobacillus* and *Enterobacteria* species abundance after weaning but interacted with a significant ($P < 0.05$) effect of litter of origin. Litter was also significant ($P \leq 0.05$) in *Bifidobacterium species* and total bacteria counts. Cluster analysis based on TRFLP banding patterns and diversity index analysis both showed no significant effect of treatment due to a low number of total animals available. This research demonstrated that microbial succession can be altered by the snatch farrow, isolator rearing and differential inoculation protocol employed despite difficulties in determining major intestinal microbial differences due to treatment postweaning.

5.2. Introduction

Microbial exposure in the early postnatal period has been suggested to have a significant impact on both adult gut microbiota composition, and host-microbiota interactions (Tannock, 2005; Mulder *et al.*, 2009). Though detailed knowledge regarding the pattern of microbial succession, using culture-dependent methods, has been previously compiled for the pig (Smith, 1965; Fuller *et al.*, 1978; Pederson and Tannock, 1989; Ducluzeau, 1985; Swords *et al.*, 1993; Mikkelsen *et al.*, 2003; Inoue *et al*., 2005; Konstantinov *et al.*, 2005; Gancarčíková *et al.*, 2008), more information is needed about the effects of variation in microbial succession in the pig post weaning. From initial gut colonization at birth until gut microbial stabilization in the adult pig, the development of microbial succession has been shown to occur concurrently with the growth and maturation of the gut mucosal immune system (Kelly, 1998; Mulder *et al.*, 2011). It has been previously shown that the maintenance and maturation of immune function in the gut is directly influenced by the presence of commensal bacteria (Kelly, 1998; Kelly *et al.*, 2007; Mulder *et al.* 2009 and 2011). This is most apparent in germ-free animals where the immune system is highly underdeveloped (Macpherson *et al.* 2004 and 2005) and immune maturation can only be triggered by the introduction of intestinal or fecal material from conventionally raised animals (Ishikawa *et al.*, 2008, Lotz *et al.*, 2006; Wagner 2008, Shirkey et al. 2006; Willing *et al.* 2007). Furthermore, exposure to pathogenic bacteria during the developmental window has been linked to increased incidence of infectious, inflammatory and autoimmune diseases (Elson 2006, Kelly *et al.*, 2007). Therefore, the critical time period between birth and adult gut maturity can be seriously affected by the birth environment and microbial exposure (Kelly *et al.*, 2007; Mulder *et al.*, 2009 and 2011). On the human side, Kelly *et al.* (2007) proposed the hygiene hypothesis as a link between gut microbiota and disease. They described differences in nutrient availability and immune status observed post adolescence as associated

with differences in microbial exposure in new born children and infants. It was determined that diversity shifts and colonization differences, especially in mucosal surfaces, have long lasting impact on humans later in life, for example in regard to irritable bowel syndrome or allergy development (Kelly *et al.*, 2007).

The objective of this study was to establish a model to control early bacterial colonization pattern in the pig and examine impact on intestinal ecology and physiology post weaning. We hypothesized that rapid collection, aseptic handling, and isolator rearing of newborn piglets would limit microbial exposure and permit controlled microbial treatments during the isolator phase. Further, we hypothesized that successful differentiation of early postnatal microbial colonization using this approach, would result in differentiated microbial profiles post weaning.

5.3. Materials and Methods

5.3.1. Piglets and Experimental Design

A total of 15 piglets from each of 3 litters were used (2nd parity, PSC, Saskatoon, Canada). Sows were induced to farrow using 3 peri-vulvar injections of 5 mg prostaglandin F2α (PGF; Lutalyse, Pharmacia, Orangeville, Ontario) administered twice on 115 days of gestation, at 10.00 and 16:00 h. The following day, 20 IU oxytocin (Bimeda-MTC Pharmaceuticals, Cambridge, Ontario) was administered at 08.00 h. Prior to expected parturition, the hind quarters of the sow were cleaned with warm water, dried and rubbed with iodine solution (0.1N, Fisher Scientific, Nepean, Ontario, Canada) around the external peri-vulval region. Iodine solution was again applied preceding as closely as possible the birth of each piglet. Piglets were collected directly from the vaginal canal into a sterile towel and transferred to a sterile covered container fitted with a HEPA filter to permit air exchange. Piglets were transferred from these containers into one of six isolators at the gnotobiotic facility (University of Saskatchewan,

Saskatoon, Canada). Piglets from each litter we assigned to one of two isolators such that a total of six isolators were employed. One isolator per litter was inoculated with sow feces within 24 hours of birth and the remaining isolator left untreated. The sow feces inoculant was pooled from 6 lactating sows, mixed in 10% glycerol (Sigma-Aldrich) and stored at -80 °C prior to use. The sow feces inoculant was thawed for 2 h at room temperature and transferred into the isolator. Approximately 150 mg of sow feces inoculant per piglet was mixed with milk replacer and immediately fed to the animals.

After placement in isolators, all piglets were syringe-fed a mixture of 1.1 L sterile water, 250 g irradiated (5Mrad) spray dried bovine colostrum (HeadStart®, Saskatoon Colostrum Company Ltd, Saskatoon, SK, Canada), 50 g irradiated porcine animal blood plasma (AP 920®, APC Nutrition Ltd., Verchères, QC, Canada) and 80 mL irradiated medium-chain triglyceride oil (MCT MaxxTM, PVL Nutrients Ltd, Mississauga, ON, Canada) in 4 doses per animal of 3 mL each. Subsequently piglets were bottle fed with a 2:1(v/v) iron fortified infant milk formula concentrate (Similac ® Advance ®, Abott Laboratories Limited, Saint-Laurent, QC, Canada) to water mixture supplemented with 1% AP 920® supplemented until 2 days of age adjusted to 1.5:1 (v/v) formula to water mixture and trough feeding 4 times per day from day 2 to day 7. Fecal samples were collected from each pig at 4 days of age, followed by inoculation of all pigs with 150 mg sow feces inoculant per piglet. The inoculation procedure and sow feces inoculant were similar to day 0 as described above.

On day 7 of age, pigs were removed from isolator units and placed into two raised pens such that pens were balanced for litter and inoculation treatment (WCVM, University of Saskatchewan, Saskatoon, Canada). Room temperature was maintained at 28 °C decreasing to 20 °C in increments of 1 °C/day. A heat lamp was also placed in each pen. Light was set to a 12

hours light to 12 hours darkness cycle. Direct animal contact between pens was possible. The formula to water mixture with 1% porcine plasma was gradually replaced with non-medicated milk replacer (Wet Nurse®, Prairie Micro-Tech Inc., Regina, SK, Canada) supplemented with 1% porcine plasma. Starting on day 16, commercial non-medicated pig starter (Whole Earth Pig Start®, Federated Co-Operatives Limited, Saskatoon, SK, Canada) was introduced in increasing amounts starting with 50 g in milk replacer. On day 20 of age, piglets were abruptly weaned to pig starter supplemented with 1% porcine plasma.

Animals were humanely killed at day 28 of age by emersion in CO_2 and exsanguination to permit sample collection. All contents from stomach, small (duodenum through ileum) and large intestine (cecum and colon) were collected, homogenized and sub-sampled for commensal microbiota analysis. Digesta sub-samples were snap frozen on dry ice and stored at -80°C until processing for analysis. The experimental protocol was approved by the University of Saskatchewan Animal Research Ethics Board (Protocol Number 20070073) according to guidelines established by the Canadian Council on Animal Care (Olfert *et al.*, 1993).

5.3.2. Microbial Analysis Of Vaginal Microbiota

To monitor the exposure of piglets to the microbiota of the birth channel, vaginal swabs were taken at 15 cm of depth with 28” guarded culture swabs (Jorvet, Jorgensen Laboratories Inc., CO, USA). Swabs were directly transferred into 15 mL sterile conical vial on ice. Within 1 hour of sampling, cotton heads were removed and placed in 200 µL DNAzol ® direct reagent (Invitrogen, CA, USA) in standard 1.7 mL cryogenic vials, incubated for 15 minutes at room temperature and heated to 90 °C for 15 minutes, then stored at room temperature as described in Hill et al. (2005b). Sample collection was performed on day 110 and 114 of gestation, during farrowing between the 1st and 2nd born piglet as well as at 1 and 5 days after farrowing.

DNA extracted from sow vaginal swabs was used as template in separate PCR reactions with *chaperonin*60 universal target (*cpn*60 UT) primers (Hill *et al.*, 2005) or 16S rRNA gene specific primers F1 and R3 (Dorsch and Stackebrandt, 1992) as described in section 3.3.2 above. A range of annealing temperatures (55-63 °C), touch-down PCR program (Korbie and Mattick, 2008) and a range template concentrations were employed.

5.3.3. Molecular Analysis of Intestinal Microbiota

For digesta microbial analysis, DNA was extracted from feces collected at 4 days of age (prior to second inoculation of all pigs with sow feces) and from digesta collected at 28 days of age, as previously described (Dumonceaux et al. 2006). Quantitative PCR was performed to enumerate selected bacterial groups using primers and annealing temperatures given in Table 5.1. Per reaction, 0.02 µL each of 25 µM forward and reverse primer (Table 5.1), 7.96 µL double distilled sterilized water, 2 µL of template cDNA and 10 µL SsoFastTM EvaGreen® Supermix (BioRad) was used. Reaction conditions for qPCR were: 1 x 95 °C for 2 min, 35 x (95 °C - 5s, annealing temperature for 10s), following a dissociation curve analysis from 65 °C to 95 °C in 0.5 °C increments for 5s each. Reactions were conducted using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, Canada).

Standard curves were generated using gel purified (QIAEX II, Qiagen) amplicons prepared by standard PCR using pooled genomic DNA extracted from feces as template and primers described in Table 5.1. Standard PCR reaction conditions were: 1 x 95 °C - 3 min, 35 x (95 °C - 40s, annealing - 40s, 72 °C - 60s), 1 x 72 °C - 3 min; Amplification was performed using 5 µL of 10 x PCR buffer, 1.5 µL of 50 mM MgCl2, 1 µL of 10 mM dNTP mix, 1 µL of 10 mM

Table 5.1. Annealing temperatures and primers used for quantitative PCR in chapter 5.

Name	Target	Sequence (5'-3') [1]	Ann. (°C)[2]	Amp. (bp)[3]	Citation
TotBac	16S rRNA gene V3 region, domain bacteria	CGGYCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	60	200	Lee *et al.*, 1996
Ccl1	16S rRNA gene, genus *Clostridium* cluster I	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	63	346	Song *et al.*, 2004
Lacto	16S rRNA gene, genus *Lactobacillus*	GCAGCAGTAGGGAATCTTCCA GCATTYCACCGCTACACATG	55	346	Walter *et al.*, 2001
Strepto	16S rRNA gene, genus *Streptococcus*	AGAGTTTGATCCTGGCTCAG GTTAGCCGTCCCTTTCTGG	57	485	Nübel *et al.*, 1996 Franks *et al.*, 1998
Entero	16S rRNA gene, family Enterobacteriaceae	ATGGCTGTCGTCAGCTCGT CCTCATTCTTTTGCAACCCACTC	60	364	Castillo *et al.*, 2006
Bifido	16S rRNA gene, genus *Bifidobacterium*	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	56	563	Matsuki *et al.*, 2002

[1] top forward, bottom reverse; [2] Annealing temperature in °C; [3] Amplicon size in base pairs.

primer (each), 2 µL of 1 µg/µL of bovine serum albumin, 0.5 µL Taq polymerase, 2 µL template DNA, 36 µL dd UV H2O (50 µL total volume, all solutions from BioRad). Amplicons were quantified by O.D.260nm and 6 serial 1/10 dilutions prepared starting with 5 pg / µl concentration and including a no DNA template control. Standards were converted to copy numbers using the formula:

Number of copies = [DNA (pg) * 6.022x1023 (copies per mole)] / [fragment length (bp) * 1x1012 (pg / g) * 650 (g / mole of bp)]

All amplifications were followed by melt curve analysis (BioRad CFX manager software, version 1.6.541.1028) to ensure single product amplification (Figures 5.1). Duplicate threshold cycles (Ct) were averaged and mean values with greater than ± 0.50 standard deviations were reanalyzed. Acceptable reaction efficiency (E) was set for the range 0.90 - 1.10, and standard curve R2 values to≥ 0.97 (Figures 5.1). Results were expressed $\log_{10}$ (number of gene copies) per g of content using formula below:

Number of copies / g of content = [Vol. after DNA extraction (µL) / IW content (g)] x [Dilution Vol. (µL) / Vol. aliquot in dilution (µL)] x [Number of copies / aliquot for qPCR reaction (µL)]

Calculation of bacterial group abundance as a percent of total bacteria was accomplished as described in chapter 3 (section 3.3.3).

Terminal-restriction fragment length polymorphism (TRFLP) analysis was performed as described in Fernando et al. (2010). Primers 8F [FAM(carboxylfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide) - AGAGTTTGATCCTGGCTCAG; Fernando et al., 2010] and R3 (TCTACGCATTTCAC; Dorsch and Stackebrandt, 1992) were used in two standard PCR reactions with the following conditions: 1 x 95 °C - 3 min, 35 x (95 °C - 40s, 50

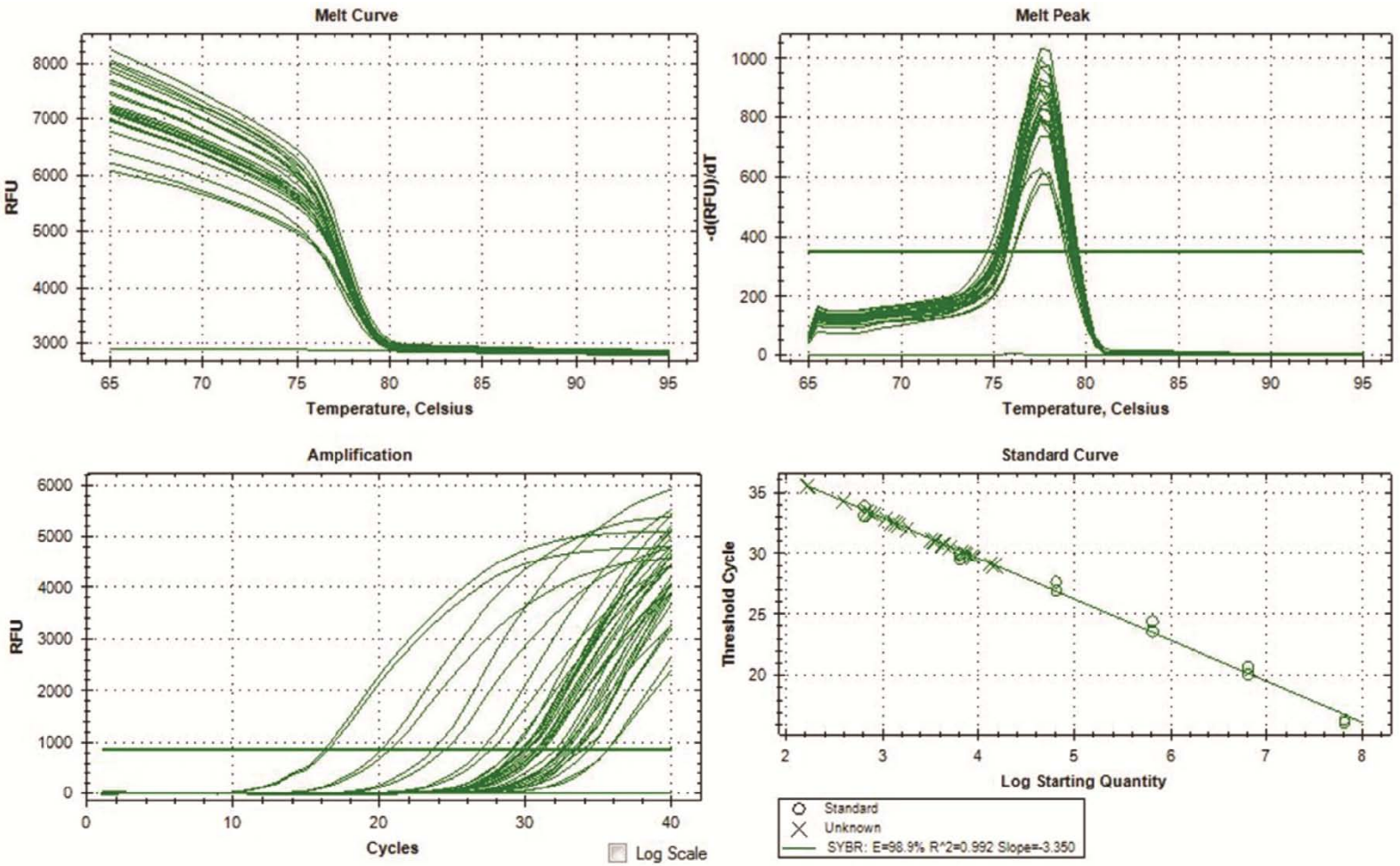

Figure 5.1 Representative output from BioRad CFX manager software used to assess performance of qPCR analysis for each bacterial group target. Panels a and b show melt curve analysis for standards and samples indicating a single amplification product.Panel c shows the relative florescence units (RFU) plotted against PCR cycle for standards and samples. The solid line in panel d is a regression of threshold cycle and starting quantity (log_{10} 16S rRNA gene copies per reaction) for standards (open circles). Samples in panel d are represented by crosses.

°C - 40s, 72 °C - 60s), 1 x 72 °C - 3 min. Each reaction was in a total volume of 50 µL performed using 5 µL of 10xPCR buffer, 1.5 µL of 50 mM MgCl2, 1 µL of 10 mM dNTP mix, 1 µL of 10 mM primer (each), 2 µL of 1 µg/µL of bovine serum albumin, 0.5 µL taq polymerase and 2 µL template DNA (all solutions from BioRad). Quality (single product of expected size) of pooled labelled PCR product was confirmed by gel (1.5% agarose) electrophoresis of a 3 µL aliquot and, for quality confirmed products, the remainder was purified (QIAquick spin column purification kit, Qiagen, CA, USA) and eluted in a final volume of 30 µL double-distilled UVed H2O. Purified PCR products were quantified by O.D.260 nm and restriction digests were performed in triplicate with 100 ng of purified DNA in 20 µL reactions with MspI, HaeII or HhaI (source) while incubating at 37 °C for 5h. Aliquots of 2 µL of restricted DNA were each combined with 9 µL deionized formamide and 0.5 µL 600 LIZ internal size standard (Applied Biosystems, CA, USA), denaturated at 95 °C for 2 minutes and stored at -20 °C prior to analysis. Terminal Restriction Fragment (TRF) lengths were determined by ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA), TRFLP profiles analyzed by GeneMapper software version 3.7 (Applied Biosystems, CA, USA) and TRF size in base pairs (bp; range from 25 – 600 bp) estimated by Local Southern method using LIZ standard. Sample data consisted of TRF size in base pairs and related peak area. Relative peak area was determined for each sample replicate by calculating the sum of all TRF peak areas and recording each TRF as a percent of total peak area. Relative peak areas for 3 replicates were averaged for each sample and relative peak areas below 1% removed from analysis to reduce background noise. Relative data was transformed by formula:

Normalized relative peak area = [arc sin √ (relative value)] (Fernando et al., 2010).

Normalized TRF profiles were imported into Bionumerics software version 5.1 (Applied Maths, TX, USA). Band based fingerprint UPGMA cluster analysis was performed using Dice (band presence/absence) and Jaccard (band presence/absence balanced for band intensity) option as well as 1% position tolerance. In addition to that, bacterial community diversity was determined using Shannon-Weiner index (Shannon and Weaver, 1949) based on relative peak areas of MspI TRFLP profiles of individual samples.

Table 5.2 shows the assignment of bacterial species to terminal restriction fragments (TRFs) established using an *in silico* digest of library sequences reported in Chapter 3 and the online T-DistinctiEnz tool (http://www.bioinformatics.org/~docreza/cgi-bin/restriction/t_DistinctiEnz.pl; Bioinformatics Organization Inc., MA, USA). Restriction enzymes MspI, HaeIII and HhaI were used for restriction digest. Since MspI digest resulted in best discrimination of species groups, analysis of relative TRF abundance was conducted for MspI data only.

5.3.4. Statistical Analysis

Data was analyzed as a complete randomized block design with litter as block and animal as the experimental unit using Poc Mixed procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA) with significance level $\alpha = 0.05$. A gender effect was originally tested but removed from the model since gender and its interactions were not significant. Data analysis was performed independently for each location. Means were separated by Least Significant Difference (LSD) for block effects.

Table 5.2. Assignment of Terminal Restriction Fragments (TRFs) to a bacterial species based on comparison of the observed TRF size compared to that predicted by *in silico* digest of intestinal library sequences from neonatal pig reported in Chapter 3.

Accession # or Reference	Species	Observed (bp)	Predicted (bp)
HQ 701510	*L. antri*	28	28
HQ 701638	*L. mucosae*	28	28
HQ 701597	*L. pontis*	28	28
HQ 701651	*L. vaginalis*	28	28
HQ 701486	*B. fragilis*	43	43
HQ 701169	*S. pyogenes*	48	48
HQ 701443	*R. lactaris*	75	75
HQ 701477	*B. fragilis*	99	99
HQ 701148	*S. simulans*	146	147
HQ 701318	*L. delbrueckii*	152	152
HQ 701310	*L. crispatus*	155	155
HQ 701401	*L. amylovorus*	178	180
HQ 701418	*L. crispatus*	178	179
HQ 701582	*L. gasseri*	188	188
HQ 701609	*L. johnsonii*	188	188
HQ 701170	*E. fergusonii*	194	194
HQ 701614	*C. hathewayi*	204	204
HQ 701602	*C. amygdalinum*	220	220
HQ 701356	*C. nexile*	220	220
HQ 701601	*C. symbiosum*	220	220
NR_044624	*C. cellulosi* [1]	281	281
NR_074399	*R. albus* [1]	281	282
AJ270470	*F. prausnitzii* [1]	284	284
NR_044644	*E. desmolans* [1]	296	296
HQ 701516	*V. caviae*	300	301
HQ 701548	*A. polyendosporus*	371	371
HQ 701180	*S. infantarius*	460	460
HQ 701475	*M. canis*	483	484
HQ 701515	*P. stomatis*	483	484
HQ 701172	*S. dysenteriae*	494	495
HQ 701159	*S. flexneri*	494	495
HQ 701278	*A. porcinus*	497	497
HQ 701161	*C. subterminale*	516	516
HQ 701147	*C. celatum*	520	519
HQ 701306	*C. colicanis*	520	521
HQ 701168	*C. disporicum*	520	521
HQ 701186	*C. perfringens*	520	521
HQ 701556	*S. gallolyticus*	553	554
HQ 701579	*S. hyointestinalis*	553	554
HQ 701344	*S. suis*	553	554
HQ 701497	*L. salivarius*	566	567

Accession # or Reference	Species	Observed (bp)	Predicted (bp)
HQ 701415	*L. manihotivorans*	571	571
HQ 701412	*L. zeae*	577	577

[1] Indicated TRFs could not be associated with species from sequence library established in Petri *et al.*, 2010 due to sequence similarity ID < 97%.

5.4 Results

5.4.1. Determination Of Microbiota Flora In Birth Channel

Amplification of bacterial DNA from sow vaginal swabs with universal 16S rRNA gene and *cpn*60 UT primers was not successful, implying that either bacterial genomic DNA were below detection level or extracts contained high levels of PCR inhibitors.

5.4.2. Treatment Confirmation

Major complications during snatch farrowing occurred. The first sow farrowed within 4 hours after induction delivering 11 piglets total over a period of 2.5 hours. For the first sow, this permitted rapid processing, colostrum-replacer feeding and placement of piglets within two isolator units within 2 hours of birth. Sows 2 and 3 did not farrow until 6-8 h after induction and sow 2 had a difficult birth with a long delay between pigs 2 and 3. Both litters were unexpectedly small with 5 and 4 piglets for litter 2 and 3, respectively. Piglets of litters 2 and 3 were transferred to their respective isolators within 6 h and 4 h after birth of the first piglet, respectively.

Quantitative PCR analysis of DNA extracted from feces taken on day 4 indicated no effect of treatment on total bacteria abundance however, a clear treatment effect ($P < .001$) was observed for abundance of all bacterial groups assessed except *Clostridium* cluster I (Table 5.3). A significant effect of litter (block) was also observed for several bacterial groups and total bacteria although no pattern was identified for any single litter.

Table 5.3. Mean number of 16S rRNA gene copies (log_{10} copies/g of digesta) for major taxonomic groups recovered from feces collected at 4 days of age from snatch-farrowed, isolator-reared pigs inoculated (inoc) or not (clean) with sow feces at 0 days of age.

		TotBac	Entero	Ccl1	Strepto	Lacto	Bifido
Treatment	clean	12.11	11.07 [a]	9.49	1.84 [a]	6.63 [a]	6.26 [a]
	inoc	12.25	11.71 [b]	9.79	8.01 [b]	8.66 [b]	6.58 [b]
	P-value	*0.197*	*0.010*	*0.399*	*<0.001*	*0.001*	*0.097*
Block (Litter)	1	12.06 [a]	11.11 [a]	9.36	4.21	9.12 [b]	6.84 [b]
	2	12.38 [b]	11.55 [b]	9.96	6.27	5.47 [a]	6.04 [a]
	3	12.09 [ab]	11.51 [b]	9.60	4.29	8.34 [b]	6.38 [ab]
	P-value	*0.050*	*0.030*	*0.356*	*0.209*	*<0.001*	*0.008*
SEM [2]		0.20	0.26	0.65	1.94	0.82	0.34

[1] Data in log10 copy number / g of content, separation of means by least square difference, significance level $\alpha = 0.05$; TotBac, total bacteria; Entero, *Enterobacteria*; Ccl1, *Clostridium cluster I*; Strepto, *Streptococcus*; Lacto, *Lactobacillus*; Bifido, *Bifidobacterium*; [2] SEM, standard error of the mean.

5.4.3. Cluster and Diversity Analysis

Analysis of TRFLP banding patterns determined for digesta collected at 28 days of age indicated clustering by location only (Figure 5.2). No clustering by treatment was observed. Shannon diversity index was not significantly altered by treatment or litter, however, numerically lower values were observed for stomach and colon of non-inoculated animals (Table 5.4).

5.4.4. Quantitative Analysis Of Digesta

Overall, quantitative PCR results of major bacterial species in post-weaned, 28 day old pigs did not indicate significant differences due to treatment associated with high variability between individual animals (data not shown). Analysis of relative terminal-restriction fragment length polymorphism abundance revealed significant differences in several assigned TRFs (Table 5.5). In stomach, the TRFs assigned to *Bacteroides* spp. and to *Lactobacillus delbrueckii* were increased in relative abundance of inoculated pigs, whereas TRFs assigned to *C. subterminale* and *Streptococcus* spp. were lower in relative abundance. In colon TRFs assigned to *S. pyogenes*, *R. lactris* and *L. gasseri* were increased in relative abundance in inoculated pigs. Interestingly the TRF assigned Bacteroides that was increased in stomach of inoculated pigs was reduced in the colon of these same pigs.

5.5 Discussion

5.5.1. Model Development

That the experimental protocol for collection of piglets with minimal environmental contamination followed by isolator rearing did not result in a marked difference in microbial composition at 4 days of age is remarkable. TRFLP analysis did not detect differences in community composition or diversity. Quantification by qPCR did show significant differences

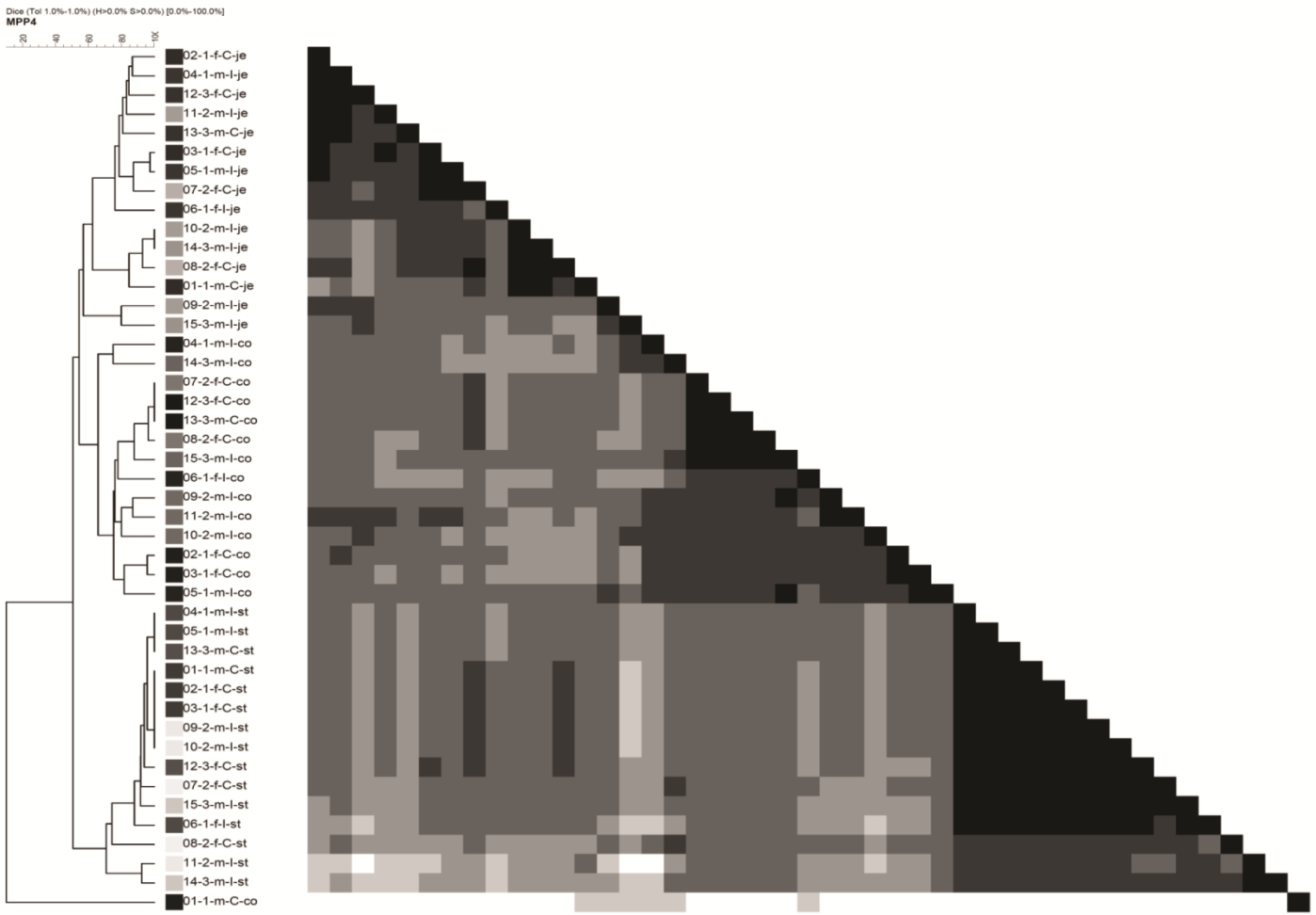

Figure 5.2. Cluster analysis of TRFLP banding patterns including similarity matrix based on digesta samples collected at day 28 of age. Sample code separated by dash: animal # (1 - 15); block (1-3, litter of origin); gender (m, male; f, female); treatment (C, clean/not inoculated on day 0; I, inoculated on day 0); location (st, stomach; je, Jejunum/mid small intestine; co, Colon). No difference in clustering was observed between Dice (see above) and Jaccard (not shown) option.

Table 5.4. Shannon diversity indices determined from TRFLP profiles generated from digesta collected from stomach, small intestine and colon of snatch-farrowed, isolator-reared pigs on day 28 inoculated (inoc) or not (clean) with sow feces at 0 days of age.

		Stomach	Small intestine	Colon
Treatment	clean	0.786	0.982	0.805
	inoc	0.867	0.963	0.992
	P-value	*0.256*	*0.812*	*0.177*
Block (Litter)	1	0.802	1.014	0.842
	2	0.837	0.945	0.924
	3	0.840	0.959	0.929
	P-value	*0.873*	*0.728*	*0.816*
SEM [1]		0.130	0.148	0.249

[1] SEM, standard error of the mean.

Table 5.5. Relative normalized peak area analysis of specific terminal restriction fragments (TRFs) analyzed as complete randomized block (RCB) design on day 28. [1]

Location_TRF (Association)		Sto_99 (*B. fragilis*)	Sto_152 (*L. delbrueckii*)	Sto_516 (*C.subterminale*)	Sto_552 (*Streptococcus*)	Co_48 (*S. pyogenes*)	Co_75 (*R. lactris*)	Co_99 (*B. fragilis*)	Co_186 (*L. gasseri*)
Treatment	clean	0.123 [a]	0.015 [a]	0.265 [b]	0.482 [b]	0.008 [a]	0.001 [a]	0.272 [b]	0.103 [a]
	inoc	0.335 [b]	0.171 [b]	0.060 [a]	0.251 [a]	0.155 [b]	0.155 [b]	0.142 [a]	0.316 [b]
	P-value	*0.010*	*0.001*	*0.006*	*0.050*	*0.034*	*0.007*	*0.047*	*0.019*
Block (Litter)	1	0.290	0.113	0.149	0.511	0.023	0.098	0.099 [a]	0.286
	2	0.185	0.102	0.222	0.286	0.039	0.043	0.238 [ab]	0.179
	3	0.212	0.064	0.118	0.302	0.184	0.087	0.285 [b]	0.162
	P-value	*0.451*	*0.556*	*0.399*	*0.172*	*0.121*	*0.619*	*0.050*	*0.375*
SEM [2]		0.130	0.071	0.114	0.205	0.118	0.095	0.110	0.148

[1] Data in relative normalized peak area, separation of means by least square difference, significance level $\alpha = 0.05$; Location_TRF, location (Sto, Stomach; Co, Colon) followed by TRF size in base pairs (bp); details on TRF association see table 5.1; clean, animals not inoculated with sow feces on day 0; inoc, animals inoculated with sow feces on day 0; SEM, standard error of the mean; qPCR results were not significantly altered by treatment; [2] SEM, standard error of the mean.

an abundance of several taxa, differences were marked for S*treptococcus* and *Lactobacillus* spp.. *Streptococcus* spp. is the most predominant genus in the GIT of the conventionally reared piglet between day 1 and 3 of age reaching over 10.3 log copies per gram cecal contents whereas *Lactobacillus* spp. become predominant by 5 days of age reaching 10.34 log gene copies per gram cecal contents (Chapter 3). Thus the abundance of these genera in the feces inoculated group reported here is consistent with conventional rearing. It is unclear why such low colonization of these two predominant genera occurred in non-inoculated pigs. Perhaps, specific predominant species within these genera were excluded by the snatch-farrow protocol and no other species were capable of occupying the vacant niche. In a similar study comparing isolator-reared pigs originated from high hygiene indoor and low hygiene outdoor farrowing systems, major differences were observed in the Lactobacillaceae family and specifically in *L. johnsonii* in pigs at day 56 of age (Schmidt *et al.*, 2011). Results presented here indicate that the host gastrointestinal environment is the dominant factor in microbial succession rather than microbial profile of the immediate environment.

It was not possible to amplify bacterial genes from DNA extracted from sow vaginal swabs. Efforts to amplify a bacterial target from vaginal swabs were extensive and included use of numerous primers designed for eubacteria and selected taxa based on both 16S rRNA gene and the *cpn*60 universal target gene. Although results could reflect a technical limitation, Bara *et al.* (1993) were unable to culture bacteria from vaginal swabs obtained from periparturient sows suggesting a porcine vaginal tract either harbors very low bacterial counts around birth. This group suggested that the reproductive tract of the sow actively inhibits vaginal microbiota due to cyclic ovarian hormonal patterns, immunoglobulin and mucus secretion, and phagocytic granulocytes (Bara *et al.*, 1993). In humans however, there is an evidence of an extensive and

abundant human vaginal flora easily assessed by molecular methods (Hill *et al.*, 2005b). Although the pig has been identified as an excellent model for studies investigating human neonatal develop and digestive physiology (Li *et al.*, 2009; Shan *et al.*, 2005), the differences in vaginal microbial colonization observed here may represent an important difference affecting microbial succession patterns.

Collection and analysis of microbiota from vaginal swabs taken from the sow was intended to permit association between the vaginal microbial composition and the GIT microbial composition in non-inoculated pigs at 4 days of age. Obviously, no associations were possible. However, the very low abundance or absent vaginal microbiota found in the sow makes the finding of a diverse microbiota in non-inoculated pigs even more remarkable.

Microbial composition at 4 days of age was affected by litter of origin. This is interesting as increased similarity in microbial profile has been reported previously in rodent littermates and in human siblings (Campbell *et al.* 2012; Palmer *et al.* 2007) but not in pig littermates (Thompson *et al.* 2008), including in our own work (Chapters 3 and 4). Similarity in microbial composition among littermates could be mediated by common genetic or environmental factors. A very limited or absent vaginal microbiota may be unique to the sow, and an environmental factor that contributes to a lack of litter effect observed previously in conventional pigs. The litter effect reported here might be more related to the variation in handling and microbial exposure encountered during collection and processing of the litters before transfer to the isolators. Because the number of piglets per isolator was low, (litter size was lower than expected), we did not investigate the effect of litter as a factor. Thompson *et al.* (2008) reported, however, that co-housing was an important factor influencing microbial composition in GIT of pigs at 7 days of age and, although litters were distributed between two isolators, co-housing may also have

contributed here. It is remarkable though that, litter effects were evident given that one half of the litter received a fecal inoculant. The suitability of the model to assess the impact of early postnatal microbial succession on postweaning colonization and health, the magnitude and extent of litter-to-litter differences in abundance of selected taxa was similar to the effect of treatment.

Many more studies comparing environmental conditions in relation to microbial succession in the gut have been accomplished in humans. For example, a study comparing breast- to formula-fed infants showed variation in *Bifidobacterium* and *Bacteroides* genera in feces (Harmsen *et al.*, 2000; Apajalahti *et al.* 2001), both of which were also altered in the current study due to the applied difference in postnatal hygiene. Schwiertz *et al.* (2003) compared the gut microbiota of pre-term infants placed in isolators to breast-fed, full term infants and revealed a high degree of similarity of gut microbiota among individual infants placed in isolators as early as 3 days post-partum. Interestingly, Enterobacteria were more commonly found in preterm infants with higher degree of hygiene compared to vaginally-delivered full term infants (Schwiertz *et al.*, 2003). This contrasts the present study where non-inoculated isolator reared pigs had fewer enterobacteria per gram feces compared to inoculated pigs. Schwiertz *et al.*, (2003) found no difference in *Streptococcus* spp. in infants due to neonatal hygiene, also in contrast to our findings.

5.5.2. Effect Of Variation In Microbial Succession On Postweaning Microbiota

It was initially hypothesized that establishing different early postnatal bacterial colonization patterns (d0 – 4) would modify colonization profile and intestinal physiology after weaning (d28), confirming implications of the early postnatal environment on intestinal health. Research by Mulder *et al.* (2009 and 2011) indicated that the hygiene environment early in piglet life significantly impacts the gut microbial composition, the mucosal innate immunity and the expression of a number of immune-related genes up to 56 d of age.

Fingerprint analysis (TRFLP) of the microbiota in intestinal contents collected at 28 d of age in the present experiment did not demonstrate clustering according to treatment as hypothesized. Obvious clustering by location within the GIT was expected as previously described using DGGE analysis (Konstantinov *et al.* 2006) providing confidence in the methodology employed. Similar to fingerprint analysis, qPCR results for major bacterial species in post-weaned, 28 day old pigs did not indicate significant differences due to treatment. Values were impacted by low number of animals per treatment and high variability between individual animals (data not shown). Though the high animal variability was similar to results described by Konstantinov *et al.* (2006), the observed variability combined with the low number of pigs and difficulty of working with farrowing sows limited our ability to assess the effect of early colonization profile with the snatch-farrow model. Despite these limitations, a clear difference in colonization at 4 d of age was seen to be associated with treatment.

Terminal-restriction fragment length polymorphism (TRFLP) analysis of individual species TRF's was the only analysis that revealed significant differences in microbiota at 28 days of age between the clean and the inoculated piglets. With the exception of the colon associated *Bacteroides* TRF, no significant litter effect was determined, which was consistent with findings of Thompson *et al.* (2008). In context of the large number of TRFs determined in the three gut locations investigated, eight TRFs have been identified post weaning on day 28 with significant differences due to neonatal hygiene. With the exception of *Streptococcus* spp., the taxa corresponding to these TRF have previously been reported to be affected by early postnatal hygiene either in human (Grölund *et al.*, 1999; Harmsen *et al.*, 2000; Apajalahti *et al.* 2001; Schwiertz *et al.*, 2003; Löfmark *et al.* 2006) and porcine studies (Thompson *et al.*, 2008; Mulder *et al.*, 2011; Schmidt *et al.*, 2011). Nevertheless, the differences in TRF abundance provide weak

evidence that the snatch-farrow and isolator rearing model employed here was effective in altering postweaning microbial ecology.

5.6. Conclusion

It was demonstrated that microbial succession could be altered by the snatch farrow, isolator rearing and differential inoculation protocol employed. However, the magnitude and extent of differences in early postnatal intestinal microbial ecology established by the experimental protocol were far less than anticipated and limited to reduced abundance of, primarily *Streptococcus* and *Lactobacillus* spp. The lack of major differences in the early postnatal microbiota was even more surprising given our failure to detect bacteria in the sow vagina. It is possible that the lack of vaginal microbiota in the sow could contribute to the absence of clustering of microbial profile by litter in pigs, in contrast to observed litter or sibling effects observed in rodents and humans respectively,

Although the snatch-farrow model was effective in differentiating the early postnatal microbial populations, major differences in intestinal microbial profile postweaning were not observed. This combined with the difficulty in predicting timing and duration of natural farrowing in the sow suggest the model is not suitable for further development to assess postweaning implications of early postnatal microbial colonization.

6.0 MICROBIAL PROGRAMMING IN PIGS: EFFECTS OF CONTROLLED SUCCESION PATTERN ON SMALL INTESTINAL MICROBIAL ECOLOGY AND HOST RESPONSE POST WEANING

6.1. Abstract

Early postnatal microbial colonization in pigs is proposed to have prolonged influence on microbial ecology and intestinal physiology affecting postweaning animal health and productivity. Using a gnotobiotic rearing model, 24 piglets derived by caesarian section were mono-associated for the first 4 days of life by oral inoculation (2×10^8 CFU/pig) with either Gram positive epithelium adherent *L. mucosae* (L), Gram positive, non-adherent *S. infantarius* (S), non-adherent toxin-producing *C. perfringens* type A (C) or Gram negative, non-adherent, non-toxin producing *E. coli* (E). Microbial composition, histology and gene expression in the upper gut were measured. Analysis of rectal swabs taken at 4 days of age confirmed monoassociation of S, C, L and E pigs. Early postnatal monoassociation with different bacterial species resulted in significant differences in post weaning microbial composition. Although the abundance of the inoculant species on day 28 in stomach content, jejunum content and mucosa was not affected by treatment, monoassociation with *S. infantarius* and *L. mucosae* resulted in an increase in the respective genera in jejunum contents, and in *Streptococcus* in jejuna mucosa. *L. mucosae* and *E. coli* inoculated animals showed the greatest growth rate, which was consistent with increased villus height to crypt depth ratio. *L. mucosae* inoculated animals also showed highest levels of digestion and immune related gene expression and the most mature pattern of mucin staining of goblet cells, whereas *E. coli* inoculated animals had generally low digestion, immune and mucin related gene expression. Low growth rate in pigs inoculated with *S.*

infantarius was associated with highest expression of *TLR4*, NFκB and *MUC2*, and lowest expression of digestive enzymes and nutrient transporters. Pigs which were monoassociated with *C. perfringens*, despite an association with a toxin producing organism, performed numerically better in growth performance than animals inoculated with *S. infantarius* and were intermediate among treatments regarding mucosal morphology measurements and gene transcript abundance. This research clearly shows that controlled early microbial succession in neonatal pigs alters post-weaning commensal microbial composition, histological parameters and gene expression in the small intestine.

6.2. Introduction

Microbial colonization of the gastrointestinal tract in the early postnatal period has been reported to impact postnatal development, and health. Microbes protect the host in a variety of ways including catabolism of toxins (Swanson *et al.* 1987) and stimulation of mucosal immunity to protect against pathogens (Bauer *et al*. 2006). Microbial composition in the gut is highly variable and impacted by numerous factors. Aside from spacial differences along the gastrointestinal tract (longitudinal and radial), major factors affecting composition of the microbiota include age (Swords *et al.*, 1993; Petri *et al.*, 2010; chapter 4), diet composition, environment, and genotype (Lallés *et al.* 2012). Diet composition is well understood to impact the microbial ecology of the pig intestine although the effects are complex and vary greatly based on carbohydrate and protein composition (Thomson *et al.* 2012; Pieper *et al.* 2012). Environmental effects in the pig, such as cohabitation, may have a greater influence on the developing host-gut microbial interaction than maternal factors (Thompson *et al.* 2008).

Due to the tremendous impact of the gut microbiota on the host, scientific interest in the factors which influence these communities has been growing as well as interest in the long term

implications on animal health and productivity (Tannock, 2005; Mulder *et al.*, 2009; Mulder *et al.* 2011). More recently, early postnatal microbial colonization has been hypothesized to have long term effects on microbial community composition and intestinal development (Mulder *et al.*, 2009; Mulder *et al.* 2011). However, the impact on the host as a result of the manipulation of the early composition of intestinal microbiota is not well understood. The potential to inadvertently trigger undesirable outcomes on the colonization process, such as a microbiota predisposing to a disease state (i.e. obesity or immunological disorders), or to engineer the establishment of a desirable microbiota, is largely unknown (Ley *et al.*, 2006; Thompson *et al.* 2008). Therefore, it is critical that the colonization process is better understood in order to determine long-term consequences for gut community structure and health. Some studies have looked at the composition of microbiota at distinct ages (Konstantinov *et al.*, 2006) but very few studies have looked at the impact of early monoassociation on community dynamics on the establishment of an individual's intestinal microbiota and host expression of immunity and nutrient transport related genes. We hypothesized that differences in early microbial succession in neonatal pigs alters post-weaning commensal microbial composition, and small intestinal physiology. A gnotobiotic model was employed to precisely control early postnatal succession pattern and permit clear association between early colonizing bacterial species and postweaning responses.

6.3. Materials and Methods

6.3.1. Experimental Design and Sample Collection

Twenty-four germ free piglets were derived by cesarean section of 2 sows on day 114 and 113 of gestation (WCVM, University of Saskatchewan, Saskatoon, SK, Canada). Each piglet was fed 12 mL of a mixture of 1.1 L sterile water, 250 g irradiated (5Mrad) spray dried bovine

colostrum (HeadStart®, Saskatoon Colostrum Company Ltd, Saskatoon, SK, Canada), 50 g irradiated porcine animal blood plasma (AP 920®, APC Nutrition Ltd., Verchères, QC, Canada) and 80 mL irradiated medium-chain triglyceride oil (MCT Maxx^{TM}, PVL Nutrients Ltd, Mississauga, ON, Canada) by syringe in 4 doses of 3 mL. Animals were assigned to one of four gnotobiotic isolator units (Class Biologically Clean Ltd. Madison, WI) balanced by gender and litter of origin. Piglets were bottle-fed at hourly intervals a 2: 1 (v/v) mixture of iron fortified infant formula (Similac ® Advance ®, Abbott Laboratories Limited, Saint-Laurent, QC, Canada) and water supplemented with 1% AP 920® until 2 d of age. Feeding was adjusted to 1.5:1 formula to water mixture (v/v) by ad libitum trough feeding 4 times per day from 2 to 7 days of age. Pigs within each isolator were assigned to one of four treatments groups such that each pig was inoculated with 2 x 10^8 CFU of either *L. mucosae* (L), *S. infantarius* (S), *C. perfringens* (C) or *E. coli* (E) within 12 hours post-partum by mixing the bacterium with the milk replacer formula.

On day 4 of age, all pigs were inoculated with sow feces by adding approximately 150 mg of sow feces inoculant per piglet to the milk replacer formula. The sow feces inoculant was pooled from 6 lactating sows from study described in chapters 3 and 4, mixed in 10% glycerol (Sigma) and stored at -80 °C prior to use, as utilized in chapter 5. Similar to proceedings in chapter 5, inoculant was thawed for 2 h at room temperature, then transferred into the isolator, mixed into milk replacer and immediately fed to the animals. Piglets were removed from isolators at day 7 of age and transferred to 3 raised floor pens with 8 pigs per pen such that 2 pigs from each isolator were in each pen. The 1.5:1 formula to water mixture with 1% porcine plasma was gradually replaced with non-medicated milk replacer (Wet Nurse®, Prairie Micro-Tech Inc., Regina, SK, Canada) supplemented with 1% porcine plasma. Starting on day 16, commercial un-

medicated pig starter (Whole Earth Pig Start®, Federated Co-Operatives Limited, Saskatoon, SK, Canada) was introduced in increasing amounts starting with an average of 32 g in milk replacer per pig per day. On day 20 of age, piglets were abruptly weaned to pig starter supplemented with 1% porcine plasma. Animals were humanely killed at day 28 of age by emersion in CO_2 and exsanguination to permit sample collection. Two 5 cm segments of jejunum (midpoint of small intestine) were snap frozen in liquid nitrogen and a 1 cm segment was fixed in 10% buffered formalin. All digesta were collected from stomach and jejunum, homogenized sub-sampled. Digesta and tissue samples were stored at -80 °C until processing for analysis. The experimental protocol was approved by the University of Saskatchewan Animal Research Ethics Board (Protocol Number 20070073) according to guidelines established by the Canadian Council on Animal Care (Olfert *et al.*, 1993).

6.3.2 Isolation and Culture Of Inoculants

Inoculants were isolated from neonatal pig intestinal contents described in Chapters 3 and 4. Intestinal contents were cultured aerobically on DeMan, Rogosa and Sharpe agar (MRS, Difco Laboratories, Sparks, MD, USA), MacConkey agar (Sigma-Aldrich, St. Louis, MO, USA), and tryptose sulfite cycloserine agar (TSC, Merck Inc., Whitehouse Station, NJ, USA) under anaerobic conditions at 37 °C. Individual colonies were selected, cultured overnight in MRS broth (Difco Laboratories) anaerobically and brain heart infusion broth (BHI, Sigma-Aldrich) aerobically and anaerobically at 37 °C. An aliquot of culture was retained for molecular identification by 16s rRNA gene sequence determination (see sections 3.3.2) and the remainder mixed with 30% glycerol and stored at -80 °C. Isolates with greater than 97% identity of 16s rRNA gene with the type strain for *L. mucosae, S. infantarius, C. perfringens* and *E. coli* were selected. Furthermore, *E. coli* isolate was confirmed negative for adhesin genes F4/K88, K99, 987P, F41 and F18 and enterotoxin genes LT, STa and STb using multiplex PCR as described in

Casey and Bosworth (2009). The *C. perfringens* isolate was tested for toxin genes *cpa, cpb, cpe, etx* and *iA* via multiplex PCR according to Heikinheimo and Korkeala (2005) as well as for *cpb2* toxin gene as described in Waters *et al.* (2003). The strain was positive for α-toxin gene (*cpa*) only and was thus categorized as commensal *C. perfringens* type A according to Hatheway (1990).

Prior to inoculation of pigs, frozen isolates were cultured overnight at 37 °C in MRS broth (Difco Laboratories) anaerobically for *L. mucosae*, and in BHI broth (Sigma-Aldrich) anaerobically for *C. perfringens* and aerobically for *S. infantarius* and *E. coli*. An aliquot (100 µL) was serially diluted and cultured anaerobically for *L. mucosae* enumeration on MRS agar (Difco Laboratories) and anaerobically (*C. perfringens*) as well as aerobically (*S. infantarius, E. coli*) on Tryptic Soy Agar (TSA, Difco Laboratories) with 5% sheep blood at 37 °C to determine colony counts. The remaining culture was centrifuged, re-suspended in peptone and inoculated in to milk replacer 5 min prior to feeding 12 hours after cesarean section.

6.3.3. Confirmation Of Isolator Microbial Status

On days 3 and 4 of age, prior to inoculation with feces, rectal swabs were collected from each pig to confirm mono-association with inoculated bacteria. DNA was extracted from feces and amplified using 16s rRNA gene universal primers F1 and R3 (Dorsch and Stackebrandt 1992), ligated into cloning vector pGEM T Easy (source) and transformed into *E. coli*JM109 cells as described in Section 3.3.2. For each isolator and collection day, ten JM109 colonies were selected for sequencing of the plasmid insert as described (section 3.3.2). Quantitative PCR was used as described in section 4.3.2 to enumerate, in each fecal sample collected on day 4 of age, to enumerate the inoculated bacteria (*L. mucosae, C. perfringens, S. infantarius., E. coli*) using primers from Table 4.1 (Enterobacteria group primers for *E. coli*) and the *L. mucosae* primer

from Metzler *et al.*, 2009 (*L. mucosae* primer set, forward GGCTATCACTTTGGGATGGA, reverse ATGGACCGTGTCTCAGTTCC, annealing 60 °C).

6.3.4. Digesta and Mucosa Sample Preparation For Molecular Analysis

For mucosa samples, distal small intestine and colon segments were thawed on ice, dissected longitudinally and after removing contents, the mucosa harvested by scrapping with a microscope slide. DNA was extracted from mucosa and digesta as described by Dumonceaux *et al.* (2006).

6.3.5. Terminal-Restriction Fragment Length Polymorphism Microbiota Analysis

Terminal-restriction fragment length polymorphism (TRFLP) analysis was performed on digesta samples as described in Fernando *et al.* (2010). Primers 8F (carboxylfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide=AGAGTTTGATCCTGGCTCAG; Fernando *et al.*, 2010) and R3 (TCTACGCATTTCAC; Dorsch and Stackebrandt, 1992) were used in two standard PCR reactions per sample where reactions were heated to 95 °C for 3 min followed by 35 cycles of 95 °C for 40s, 50 °C for 40s and72 °C –for 60s) and ending with 72 °C for 3 min. Reaction volume was 50 µL composed of 5 µL of 10 x PCR buffer, 1.5 µL of 50 mM MgCl2, 1 µL of 10 mM dNTP mix, 1 µL of 10 mM primer (each), 2 µL of 1 µg/µL of bovine serum albumin, 0.5 µL taq polymerase and 2 µL template DNA (all solutions from BioRad). Single product amplification of expected molecular weight was confirmed by electrophoresis and reactions were pooled, purified using QIAquick spin column purification kit (Qiagen, CA, USA) and eluted in a final volume of 30 µL double-distilled UVed H_2O. Purified PCR product was digested in triplicates using 100 ng of purified DNA in 20 µL reactions with MspI, at 37 °C for 5h. Aliquots of 2 µL of restricted DNA were each combined with 9 µL deionized formamide and 0.5 µL 600 LIZ internal size standard (Applied Biosystems, CA, USA), denaturated at 95 °C for 2 minutes and stored at -20 °C prior to analysis. Terminal Restriction Fragment (TRF) lengths

were determined by ABI PRISM 310 genetic analyzer (Applied Biosystems, CA, USA), TRFLP profiles analyzed by GeneMapper software version 3.7 (Applied Biosystems, CA, USA) and TRF size in base pairs (bp; range from 25 – 600 bp) estimated by Local Southern method using LIZ standard. TRFs were assigned to candidate bacterial species using the online T-DistinctiEnz tool (http://www.bioinformatics.org/~docreza/cgi-bin/restriction/t_DistinctiEnz.pl; Bioinformatics Organization Inc., MA, USA) and 16s rRNA gene sequence data reported in chapter 3 (Table 6.1). TRF size was derived from original TRFLP analysis in human fecal matter using the same forward and reverse primers. Raw peak area data were normalized within each sample by summing TRF peak area and calculating TRF area as a percentage of total peak area. Relative peak areas for 3 replicates were averaged for each sample and relative peak areas below 1% removed from analysis to reduce background noise. TRF profiles were imported into Bionumerics software version 5.1 (Applied Maths, TX, USA) and UPGMA cluster analysis was performed using Dice option and 1% position tolerance. The Shannon-Weiner diversity index (Shannon and Weaver, 1949) was also determined based on relative peak areas of TRFLP profiles of individual samples.

6.3.6. Quantitative Polymerase Chain Reaction Of Microbiota Groups and Species

Quantitative PCR was performed using primers and annealing temperatures as previously described in Chapter 4. For each reaction, 0.02 µL each of 25 µM forward and reverse primer

Table 6.1. Terminal restriction fragment (TRF) association.

Accession #	Group[1]	Species	Observed (bp)	Predicted (bp)
HQ 701148	-	*S. simulans*	146	147
HQ 701278	-	*A. porcinus*	497	497
HQ 701475	-	*M. canis*	483	484
HQ 701515	-	*P. stomatis*	483	484
HQ 701516	-	*V. caviae*	300	301
HQ 701548	-	*A. polyendosporus*	371	371
HQ 701486	BaPr	*B. fragilis*	43	43
HQ 701477	BaPr	*B. fragilis*	99	99
HQ 701147	Ccl1	*C. celatum*	520	519
HQ 701161	Ccl1	*C. subterminale*	516	516
HQ 701168	Ccl1	*C. disporicum*	520	521
HQ 701186	Ccl1	*C. perfringens*	520	521
HQ 701306	Ccl1	*C. colicanis*	520	521
HQ 701356	Ccl14a	*C. nexile*	220	220
HQ 701443	Ccl14a	*R. lactaris*	75	75
HQ 701601	Ccl14a	*C. symbiosum*	220	220
HQ 701602	Ccl14a	*C. amygdalinum*	220	220
HQ 701614	Ccl14a	*C. hathewayi*	204	204
NR_044624	Ccl4	*C. cellulosi* [2]	281	281
NR_074399	Ccl4	*R. albus* [2]	281	282
AJ270470	Ccl4	*F. prausnitzii* [2]	284	284
NR_044644	Ccl4	*E. desmolans* [2]	296	286
HQ 701159	Entero	*S. flexneri*	494	495
HQ 701170	Entero	*E. fergusonii*	194	194
HQ 701172	Entero	*S. dysenteriae*	494	495
HQ 701310	Lacto	*L. crispatus*	155	155
HQ 701318	Lacto	*L. delbrueckii*	152	152
HQ 701401	Lacto	*L. amylovorus*	178	180
HQ 701412	Lacto	*L. zeae*	577	577
HQ 701415	Lacto	*L. manihotivorans*	571	571
HQ 701418	Lacto	*L. crispatus*	178	179
HQ 701497	Lacto	*L. salivarius*	566	567
HQ 701510	Lacto	*L. antri*	28	28
HQ 701582	Lacto	*L. gasseri*	188	188
HQ 701597	Lacto	*L. pontis*	28	28
HQ 701609	Lacto	*L. johnsonii*	188	188
HQ 701638	Lacto	*L. mucosae*	28	28
HQ 701651	Lacto	*L. vaginalis*	28	28

Table 6.1. Terminal restriction fragment (TRF) association.

Accession #	Group[1]	Species	Observed (bp)	Predicted (bp)
HQ 701169	Strepto	*S. pyogenes*	48	48
HQ 701180	Strepto	*S. infantarius*	460	460
HQ 701344	Strepto	*S. suis*	553	554
HQ 701556	Strepto	*S. gallolyticus*	553	554
HQ 701579	Strepto	*S. hyointestinalis*	553	554

[1] Entero, Enterobacteria; Ccl1, *Clostridium* cluster I species; Lacto, *Lactobacillus* species; Strepto, *Streptococcus* species; Bifido, *Bifidobacterium* species; BaPr, *Bacteroides* and *Prevotella* species; Ccl4, *Clostridium* cluster IV species, Ccl14a, *Clostridium* cluster XIVa species; [2] Observed TRFs were not identified with ≥ 97% similarity in sequence libraries of suckling pigs (Petri *et al.* 2010).

(Table 4.1), 7.96 µL double distilled sterilized water, 2 µL of template cDNA and 10 µL SsoFastTM EvaGreen® Supermix (BioRad) was used. Reaction conditions for qPCR were 95 °C for 2 min followed by 35 cycles at 95 °C for 5s, annealing temperature for 10s. A dissociation curve analysis was conducted at the end of each amplification by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments at 5s intervals. Enumeration of bacteria groups and species was conducted using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, Canada). Standard curves were generated using gel purified (QIAEX II, Qiagen) amplicons prepared by standard PCR with pooled extracted DNA as template using primers described in Table 4.1. Amplicons were quantified spectrophotmetrically ($O.D._{260nm}$) and 6 serial 1/10 dilutions prepared starting with 5 pg / µL concentration and including a no DNA template control. Standards were converted to copy numbers using the formula:

Number of copies = [DNA (pg) * $6.022x10^{23}$ (copies per mole)] / [fragment length (bp) * $1x10^{12}$ (pg / g) * 650 (g / mole of bp)]

Duplicate threshold cycles (Ct) were averaged and mean values with greater than ± 0.50 standard deviations were reanalyzed. Acceptable reaction efficiency (E) was set for the range 0.90 - 1.10, and standard curve R^2 values to $\geq$ 0.97. Results were expressed in number of gene copies per gram of content or per gram of mucosa using formula below:

Number of copies / g of content or mucosa = [Vol. after DNA extraction (µL) / IW content (g)] x [Dilution Vol. (µL) / Vol. aliquot in dilution (µL)] x [Number of copies / aliquot for qPCR reaction (µL)]

6.3.7. Histological Analysis

For histological evaluation of small intestine cross sections, tissue was fixed immediately after dissection in 10% neutral buffered formalin. Routine processing and staining was performed (Prairie Diagnostic Services, Saskatoon, SK, Canada) according to procedures

previously described for Gill's hematoxylin and eosin (H&E) and combined with 1% Alcian blue pH 2.5 staining with periodic acid and Schiff base reaction (AB2.5/PAS) (Mikel 1999; Law *et al.*, 2007). Histological analysis was performed with an Axiostar plus light microscope and AxioVision (version 3.1.2.1) measurement software (Carl Zeiss Ltd., Toronto, ON, Canada). Villus height and crypt depth were determined on H&E stained sections, using the mean measurement for 10 well oriented villi and crypts per animal. Goblet cells were enumerated for slides generated from similar sections processed with AB2.5/PAS stain over the same 10 villi and crypts, respectively. Goblet cells were categorized into mostly acidic mucus secreting, blue stained goblet cells containing sialylated, carboxylated and sulfated mucins, and purple stained goblet cells containing a mix of blue acidic and red stained α-glycol-rich neutral mucins (Fontaine and Meslin, 1994). Red stained goblet cells containing neutral mucin only could not be detected. Slides were analyzed by blinded observer.

6.3.8. Host Gene Expression Analysis

Harvested jejunal tissue was pulverized with a clean, disinfected, RNAse treated (RNaseZap® Wipes, Applied Biosystems, Streetsville, ON, Canada) and liquid nitrogen cooled mortar and pestle. Ribonucleic acid was extracted under liquid nitrogen from an approximate 35 mg homogenized subsample using the RNeasy® Mini Kit (Qiagen, Montreal, QC, Canada). Resulting RNA was quantified spectrophotometrically ($O.D._{260nm}$) using a ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and immediately further processed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Complimentary DNA was stored at -80 °C until processing for analysis. Quantitative PCR was performed using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, QC, Canada). Per reaction, 0.02 µL each of 25 µM forward and reverse primer (Tables 4.1, 6.2 - 6.4), 7.96 µL double distilled sterilized water, 2 µL of

template cDNA and 10 µL SsoFast™ EvaGreen® Supermix (BioRad) was used. Reaction conditions for qPCR were95 °C for 2 min followed by 35 cycles of 95 °C for 5s and appropriate annealing temperature as listed in Tables 6.2 – 6.5 for 10s. Following amplification, a dissociation curve analysis was conducted by increasing temperature from 65 °C to 95 °C in 0.5 °C increments for 5s each. For primer development, online Primer 3 software based NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC= NcbiHomeAd; Rozen and Skaletsky, 2000) with option Refseq mRNA was used referring to sus scrofa database (taxid:9823). For confirmation of targeted amplicon and establishment of quantification standards, PCR product was gel purified (QIAEX II Agarose gel DNA extraction kit, Qiagen), quantified spectrophotometrically, sequenced (Sanger *et al.*, 1977) and blasted using NCBI nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with option Refseq mRNA was used referring to sus scrofa database (taxid:9823) as before. For quantification, 1/10 dilutions of purified PCR product were created, starting with 1 x 10^7 target gene copies. Duplicate threshold cycle (Ct) mean values with greater than ± 0.50 standard deviations were reanalyzed. Acceptable reaction efficiency (E) was set for the range 0.90 - 1.10, and standard curve R^2 values to≥ 0.97. Housekeeping genes glycerald ehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-actin (*ACTB*) were tested. Although other researchers found *ACTB* generally to be more stable between different tissues (Nygard *et al.*, 2007; Piórkowska *et al.*, 2011), *GAPDH* gene expression showed less variability between animals compared to *ACTB* gene expression. Abundance of gene of interest were therefore expressed relative to *GAPDH* transcript abundance.

Table 6.2. Primers used for qPCR quantification of housekeeping and mucus genes.

Short Name	Sus scrofa mRNA target description (NCBI accession #)	Ori.[1]	Sequence (5'-3')	Temp.[2]	Amplicon Size	Source
ACTB	Beta-actin(DQ845171)	f r	CACGCCATCCTGCGTCTGGA AGCACCGTGTTGGCGTAGAG	63 °C	100 bp	Nygard *et al.*, 2007
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (NM_001206359.1)	f r	GTTTGTGATGGGCGTGAAC ATGGACCGTGGTCATGAGT	55 °C	129 bp	Willing and Van Kessel, 2007
Muc1	Mucin 1, cell surface associated(AY24350801)	f r	CGGAAGCAGGCACCTATAAC TCACGGCTGCTTTCTTGACA	55 °C	194 bp	Malik, 2009
Muc2	Mucin 2, not cell surface associated (AK231524.1)	f r	CGGCTCTCCAGTCTACTCGT CTCACAACGTTCTTCACGGT	55 °C	168 bp	Malik, 2009
Muc4	Mucin 4, cell surface associated (XM_001926442.1)	f r	TCACACTTCCACAGGCACTCG CCCGGAGAGAGGAACCGAGG	57 °C	182 bp	This publication
Muc13	Mucin 13, cell surface associated(NM_001105293.1)	f r	GGTGATTGCATTCGTCCTCT TAGGGATTCTGTGGCTGATC	55 °C	189 bp	This publication
Muc20	Mucin 20, cell surface associated (NM_001113440.1)	f r	ACTGCAGGTGCTTACGCCTC AGGGTCCCACTGCCATGTCT	57 °C	226 bp	This publication

[1] Ori., orientation; [2] Temp., annealing temperature;

Table 6.3. Primers used for qPCR quantification of selected brush border hydrolases and nutrient tranporter genes.

Short Name	Sus scrofa mRNA target description (NCBI accession #)	Ori.[1]	Sequence (5'-3')	Temp.[2]	Amplicon Size	Source
APN	Alanyl aminopeptidase (NM_214277.1)	f r	CAATATGCCGCCCAAAGGTTC CCGGATCAGGACGCCATTT	55 °C	200 bp	Willing and Van Kessel, 2008
PepT1	Solute carrier family 15 oligopeptide transporter (NM_214347.1)	f r	GGGTTGCTCCTCGCCGTCTG AGGAGGCCCGGGCACATTCT	60 °C	222 bp	This publication
LPH	Lactase-phlorizin hydrolase-like enzyme(XM_003359429.2)	f r	CCAAGTTCTACGCCTCCATAGTC TCCAAGAAGCAGAAGAGCAAAGA	55 °C	163 bp	Willing and Van Kessel, 2008
SGLT1	Solute carrier family 5 Sodium/glucose cotransporter 1 (NM_001164021.1)	f r	AAAGGAGAGGTCTGGGATGGTAA ATTTCCCTAGTGGCCTGAGATTG	60 °C	77 bp	Moran *et al.*, 2010
SGLT3	Solute carrier family 5 low affinity glucose cotransporter (NM_214182.1)	f r	TGGCCTCTCTCATGAGCTCCCT TGCAATTGGAGGCCCGAGGT	58 °C	245 bp	This publication

[1] Ori., orientation; [2] Temp., annealing temperature.

Table 6.4. Primers used for qPCR quantification of immune response-related genes via TRAF6 mediated pathway.

Short Name	Sus scrofa mRNA target (NCBI accession #)	Ori.[1]	Sequence (5'-3')	Temp.[2]	Amplicon Size	Source
TLR2	Toll-like receptor 2 (NM_213761.1)	f r	GACTGGCCGGAGAACTACCT TGAGCAGGAGCAGCAGGAA	55 °C	145 bp	Willing and Van Kessel, 2007
TLR4	Toll-like receptor 4 (NM_001113039.1)	f r	TCACTACAGAGACTTCATTCCCG GGTCTGGGCAATCTCATACTCAA	55 °C	148 bp	Willing and Van Kessel, 2007
NFKB1	Nuclear factor κ-B 1 (NM_001048232.1)	f r	GTGTGAAGGCCCCTCCCATG GGTTTGCAAAGCCGACCACC	57 °C	236 bp	This publication
NFKB2	Nuclear factor κ-B 2 (XM_001925704.1)	f r	GCCGGGAGAGAATCCGGAAC ATCGGAATCGGAAGCCTCGC	57 °C	276 bp	This publication
NFKBIA	NF-κ-B inhibitor α (NW_003535218.1)	f r	CCATGGAAGTGGTCCGCCAA GCCCAGGTAGCCATGGATC	57 °C	323 bp	This publication
IL-1β	Interleukin 1 β (NM_214055.1)	f r	CTCCCATTTCTCAGAGAACCAAG GTGATGGCTAACTACGGTGACAA	55 °C	113 bp	Willing, 2007
IL-6	Interleukin 6 (NM_001252429.1)	f r	AGACAAAGCCACCACCCCTAA CTCGTTCTGTGACTGCAGCTTATC	57 °C	70 bp	Smith *et al.*, 2011
IL-8	Interleukin 8 (NM_213867.1)	f r	GGACCAGAGCCAGA GGTGTGGAATGCGTATTTATGC	55 °C	174 bp	This publication
IL-10	Interleukin 10 (NM_214041.1)	f r	CCATGGAAGTGGTCCGCCAA GCCCAGGTAGCCATGGATC	55 °C	104 bp	Willing, 2007
TNFα	Tumor necrosis factor α (NM_214022.1)	f r	TGGCCCAAGGACTCAGATCAT TCGGCTTTGACATTGGCTACA	55 °C	76 bp	Willing and Van Kessel, 2007
PCNA	Proliferating cell nuclear antigen (XM_003359883.1)	f r	TACGCTAAGGGCAGAAGATAATG CTGAGATCTCGGCATATACGTG	58 °C	192 bp	Willing and Van Kessel, 2007

[1] Ori., orientation; [2] Temp., annealing temperature.

Table 6.5. Primers used for qPCR quantification of immune response-related genes via TRAF3 mediated pathway.

Short Name	Sus scrofa mRNA target (NCBI accession #)	Ori.[1]	Sequence (5'-3')	Temp.[2]	Amplicon Size	Source
IRF3	Interferon regulatory factor 3 (NM_213770.1)	f r	AAGCATTGCGTTTAGCAGAG GTCTTCGTGGGTATCAGAGG	57 °C	152 bp	This publication
IFNγ	Interferon-γ (NM_214022.1)	f r	TCTAACCTAAGAAAGCGGAAGAGAA TTGCAGGCAGGATGACAATTA	62 °C	81 bp	Smith *et al.*, 2011
FasL	Fas ligand (NM_213806.1)	f r	AAGAAGAAGAGGGACCACAATG CTTTGGCTGGCAGACTCTCT	59 °C	149 bp	Willing and Van Kessel, 2007

[1] Ori., orientation; f, forward; r, reverse; [2] Temp., annealing temperature.

6.3.9. Statistical Analysis

Using piglet as an experimental unit, data was analyzed separately for each intestinal location. Prior to statistical analysis, microbial qPCR data was $\log_{10}$ transformed, and TRFLP relative peak area was normalized using the formula:

Normalized relative value = [arc sin $\sqrt{}$ (relative value)] (Fernando *et al.*, 2010)

Preliminary analysis was performed in a 2x2x4 factorial ANOVA with factors gender, litter of origin and treatment. Since factors gender, litter of origin and their corresponding interactions were not significant in data presented here, these factors were removed from the model. For final analysis, data was analyzed as one-way ANOVA using Proc mixed procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA) and Tukey HSD for treatment mean separation. Pairwise Pearson correlation analysis was performed using PROC CORR procedure of SAS. For all tests, significance was declared at $\alpha \leq 0.05$, trends were indicated for $0.10 \geq \alpha > 0.05$.

6.4 Results

6.4.1 Confirmation of Microbial Status

The DNA sequence of 10 amplicons per treatment group prepared from genomic DNA extracted from fecal samples collected on day 4 and amplified with eubacterial 16s rRNA gene primers were 100% identical to the corresponding inoculated bacterial strain with one exception. For the L treatment, one of the 10 sequences was identified as *E. coli* (97.2% identical to the strain inoculated in treatment E) while remaining 9 sequences were identical to *L. mucosae* (Table 6.6). Quantification of inoculated bacteria using species specific primers (except in case of *E. coli*, where enterobacteria primers were employed) indicated a range of colonization levels from 6.5 $\log_{10}$ rRNA gene copies/g feces for *C. perfringens* to 11.8 $\log_{10}$ rRNA gene copies/g feces for *S. infantarius* (Table 6.6). Although a single enterobacteria sequence was detected for

Table 6.6. Abundance of 16S rDNA gene sequences (log_{10} gene copies/g) determined via quantitative PCR from fecal matter collected within isolators from individual pigs on day 4 before exposure to sow feces.

Treatment	Determined sequence	Quantity
S	*S. infantarius*	11.8 ± 0.4
C	*C. perfringens*	6.5 ± 0.2
E	*E. coli*	9.8 ± 0.3
L	*L. mucosae*	9.2 ± 0.4

L. mucosae, no enterobacteria genomic DNA was detected by qPCR. Similarly, enterobacteria could not be detected by qPCR in DNA extracted from fecal samples collected on day 3 (not shown). Furthermore, before opening of the isolators, swabs were taken inside the isolators, transferred in sterile sampling tubes containing media and cultured aerobically and anaerobically as indicated in section 6.3.2. In treatment L isolators, growth was observed on anaerobic MRS plates with single colony morphology only. Although it is possible that *E. coli* contamination of *L. mucosae* inoculated pigs may have occurred at very low abundance, we consider it most likely that the single clone identified was a technical contamination.

6.4.2. Animal Growth Rate

To minimize the risk of microbial contamination, animal body weight was not recorded until pigs were removed from the isolators on day 7 of age. Body weight (BW) at 7 days of age was not affected by treatment, however, pigs from treatment S had lower (P = 0.01) BW at 28 d of age and reduced (P < 0.01) average daily gain (ADG) from 7 to 28 d of age compared with animals in treatments E and L. Treatment C animals were intermediate in BW and ADG at 28 d of age (Table 6.7). Factors including litter of origin, gender and their interactions did not affect growth animal growth rate.

6.4.3. Microbial Analysis of Stomach Contents

Analysis of TRFLP banding patterns from stomach contents collected at 28 d of age revealed no treatment specific clustering. A trend (P = 0.07) towards higher diversity in the stomach digesta of pigs associated with treatment S was observed such that the Shannon index (averages ± standard error) for S pigs was 0.745± 0.052 compared with 0.535 ± 0.049, 0.637 ± 0.046 and 0.622 ± 0.049 for treatments E, C and L, respectively. There was no difference observed (P >0.10) in relative abundance of inoculant species containing TRFs 28 (*L. mucosae* and at least three other *Lactobacillus* spp.), 460 (*S. infantarius* and other *Streptococcus* spp.), 495 (*E. coli*

Table 6.7. Mean body weight (BW) ± standard error (SE) at 7 and 28 d of age, and Average Daily Gain (ADG) from 7 to 28 d of age for pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.

Factor		N	d7 BW(kg)	*P-value* [1]	d28 BW(kg)	*P-value* [1]	ADG(g/d)	*P-value* [1]
Treatment	S	6	2.17 ± 0.19	*> 0.10*	4.42 ± 0.28 [a]	*0.01*	107.2 ± 11.9 [a]	*< 0.01*
	C	6	2.17 ± 0.22		5.13 ± 0.51 [ab]		140.9 ± 21.0 [ab]	
	E	6	2.29 ± 0.11		5.37 ± 0.19 [b]		146.9 ± 5.1 [b]	
	L	6	2.23 ± 0.15		5.43 ± 0.33 [b]		148.0 ± 9.6 [b]	
Gender	Male	12	2.38 ± 0.11	*> 0.10*	5.40 ± 0.30	*> 0.10*	144.0 ± 11.8	*> 0.10*
	Female	12	2.18 ± 0.12		4.90 ± 0.25		129.6 ± 8.7	
Litter	Sow 1	16	2.17 ± 0.10	*> 0.10*	5.02 ± 0.20	*> 0.10*	135.7 ± 9.1	*> 0.10*
	Sow 2	8	2.20 ± 0.18		5.10 ± 0.45		138.2 ± 15.3	

[a-b] Values in same column with different superscripts are different ($P < 0.05$); [1] For all interactions $P > 0.10$.

and other enterobacteria) and 520 (*C. perfringens* and at least four other *Clostridium* spp.) in stomach contents. Due to low sequence variation within the Enterobacteriaceae family, detection of the *E. coli* inoculant using 16S rRNA gene at the species level was not possible via qPCR. Species specific primer sets were, however, designed to determine abundance of the other inoculants, *C. perfringens, L. mucosae* and *S. infantarius.* In stomach content on day 28, none of the bacterial inoculation species were significantly altered ($P > 0.10$) due to treatment (Table. 6.8), although there was high individual variability in inoculant species abundance. The abundance of inoculant species within the stomach was equal to or below 0.1% of total bacteria for all species.

The relative abundance of selected TRFs associated with major bacterial groups is given in Table 6.9 whereas qPCR enumeration of selected major bacterial groups is given in Table 6.10. Generally, the relative abundance of TRFs and corresponding bacterial group copy number determined by PCR were similar. However, enterobacteria and *Clostridium* cluster IV-associated TRFs were not detected via TRFLP analysis (Table 6.9). The lowest abundance of *Lactobacillus* spp. was observed in stomach of treatment E via both, TRFLP ($P = 0.01$) and qPCR analysis ($P = 0.07$). Abundance of *Streptococcus* spp. in stomach contents from pigs in treatment S tended to be lowest ($P = 0.10$) using TRFLP analysis, and numerically lowest ($P > 10$) by qPCR comparison. *Bifidobacterium* spp. were detected in highest ($P = 0.08$) levels in treatment L. *Bacteroides* and *Prevotella* were significantly higher ($P = 0.01$) in treatments E and L based on TRF relative abundance only. Clostridia in stomach digesta were not affected by treatment at 28 days of life using either analysis method.

Table 6.8. Mean relative abundance (% of total bacteria) of inoculated bacteria determined by qPCR in stomach contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	Cperf [2]	Sinfa [3]	Lmuco [4]
S	0.104	0.060	0.259
C	0.042	0.102	0.482
E	0.057	0.081	0.833
L	0.049	0.079	0.263
P-Value	*>.100*	*>.100*	*>.100*
SEM [5]	0.057	0.022	0.110

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of digesta, total bacteria $\log_{10}$ count/g of digesta 11.19 (S), 10.85 (C), 11.28 (E), 11.77 (L), $P > 0.10$, SEM 0.31; [2] Cperf, *Clostridium perfringens*; [3] Sinfa, *Streptococcus infantarius*; [4] Lmuco, *Lactobacillus mucosae*; [5] SEM, standard error of the mean.

Table 6.9. Mean relative normalized peak area for selected TRFs measured in stomach contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	TRF 194, 494 (Entero) [2]	TRF 516, 520 (Ccl1) [3]	TRF 28, 152, 155, 178, 188, 566 (Lacto) [4]	TRF 48, 460, 553 (Strepto) [5]	TRF 43, 99 (BaPr) [6]	TRF 281, 284, 296 (Ccl4) [7]	TRF 75, 204, 220 (Ccl14a) [8]
S	n/d	0.72	21.21$^{(b)}$	29.16 $^{(a)}$	1.30 ^{ab}	n/d	1.87
C	n/d	0.97	21.19$^{(b)}$	46.37 $^{(b)}$	0.83 ^{a}	n/d	1.89
E	n/d	1.07	2.33$^{(a)}$	42.41 $^{(ab)}$	1.71 ^{b}	n/d	0.33
L	n/d	0.35	11.16$^{(ab)}$	37.65 $^{(ab)}$	1.96 ^{b}	n/d	0.84
P-Value		*>.10*	*0.07*	*0.10*	*0.01*		*>.10*
SEM [9]		0.30	5.48	2.60	0.46		1.04

[1] Data in %, n/d, not detected; TRFs associated with [2] Enterobacteria, [3] *Clostridium* cluster I, [4] *Lactobacillus*, [5] *Streptococcus*, [6] *Bacteroides*, [7] *Clostridium* cluster IV and [8] *Clostridium* cluster XIVa; [9] SEM, standard error of the mean.

[a-b] Values in same column with different superscripts are different ($P<0.05$); $^{(a)-(b)}$Values in same column with different superscripts are different ($P<0.10$).

Table 6.10. Mean relative abundance (% of total bacteria) of selected major bacterial groups determined by qPCR in stomach contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups[1].

Treatment	Entero [2]	Ccl1 [3]	Lacto [4]	Strepto [5]	Bifido [6]	BaPr [7]	Ccl4 [8]	Ccl14a [9]
S	2.11	0.87	32.77 [(b)]	17.13	0.00009 [(ab)]	1.60	0.11	0.02
C	2.58	0.55	12.44 [(ab)]	49.48	0.00020 [(ab)]	1.65	0.09	0.02
E	1.14	0.28	9.09 [(a)]	35.11	0.00003 [(a)]	1.41	0.13	0.01
L	0.34	0.12	25.64 [(ab)]	24.65	0.00040 [(b)]	1.92	0.14	0.02
P-Value	*>.10*	*>.10*	*0.08*	*>.10*	*0.08*	*>.10*	*>.10*	*>.10*
SEM [10]	3.18	0.75	13.12	21.51	0.001	1.25	0.18	0.05

[1]total bacteria $\log_{10}$ count/g of digesta 11.19 (S), 10.85 (C), 11.28 (E), 11.77 (L), $P > 0.10$, SEM 0.31; [2] Entero, Enterobacteria; [3] Ccl1, *Clostridium* cluster I species; [4] Lacto, *Lactobacillus* species; [5] Strepto, *Streptococcus* species; [6] Bifido, *Bifidobacterium* species; [7] BaPr, *Bacteroides* and *Prevotella* species; [8] Ccl4, *Clostridium* cluster IV species, [9] Ccl14a, *Clostridium* cluster XIVa species; [10] SEM, standard error of the mea; [(a)-(b)]Values in same column with different superscripts are different (P<0.10).

6.4.4. Microbial Analysis of Jejunal Contents

As for cluster analysis in stomach digesta, TRFLP based banding pattern and diversity indices did not indicate treatment related clustering (data not shown). Also similar to findings in the stomach contents, inoculant species abundance in jejunum content (Table 6.11) was not significantly altered due to treatment ($P > 0.10$). However, similar to findings in chapters 3 and 4, longitudinal (stomach contents vs. jejunum contents) abundance for each species was significantly ($P < 0.05$; comparison not shown) different. Enterobacteria and *Bifidobacterium* were not detected via TRFLP (Table 6.12) but only by qPCR analysis (Table 6.13). Quantitative PCR analysis indicated enterobacteria counts were lowest ($P < 0.05$) in treatment L. *Clostridium* cluster I, *Streptococcus* and *Bifidobacterium* spp. were lowest in treatment C by qPCR, and TRFs associated with *Clostridium* cluster I and *Streptococcus* spp. were also lowest ($P < 0.05$) for the same group. *Lactobacillus* spp. were highest by TRFLP ($P = 0.01$) and numerically highest ($P > 0.10$) using qPCR for treatment L. *Bacteroides* / *Prevotella* and *Clostridium* cluster IV and XIVa in small intestine digesta were not affected by treatment.

6.4.5. Microbial Analysis of Jejunal Mucosa

Similar to stomach and jejunum digesta, qPCR inoculant strain abundance was not altered due to treatment (Table 6.14). However, radial location differences (jejunum contents vs. jejunum mucosa) were different for each inoculant species ($P < 0.05$; comparison not shown). Overall, *C. perfringens* was most abundant in the jejunum mucosa of trial pigs and accounted for approximately 1% of total bacteria, whereas *S. infantarius* and *L. mucosae* were most abundantly detected in jejunum content on day 28, both inoculant bacterial species accounting for more than 1% of total enumerated bacteria. Total bacteria counts in small intestinal mucosa were significantly lower ($P < 0.05$) with an average of 9.7 $\log_{10}$ rRNA gene copy number / g of mucosa (Table 6.15) compared with small intestine digesta which averaged 11.3 $\log_{10}$ copy

Table 6.11. Mean relative abundance (% of total bacteria) of inoculated bacteria determined by qPCR in jejuna contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	Cperf [2]	Sinfa [3]	Lmuco [4]
S	0.49	9.13	2.01
C	0.22	3.51	1.35
E	0.61	7.42	2.48
L	0.55	5.01	4.11
P-Value	*>.10*	*>.10*	*>.10*
SEM [5]	0.25	2.92	1.21

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of digesta 11.19 (S), 11.52 (C), 11.19 (E), 11.70 (L), $P > 0.10$, SEM 0.20; [2] Cperf, *Clostridium perfringens*; [3] Sinfa, *Streptococcus infantarius*; [4] Lmuco, *Lactobacillus mucosae*; [5] SEM, standard error of the mean.

Table 6.12. Mean relative normalized peak area for selected TRFs measured in jejunal contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	TRF 194, 494 (Entero) [2]	TRF 516, 520 (Ccl1) [3]	TRF 28, 152, 155, 178, 188, 566 (Lacto) [4]	TRF 48, 460, 553 (Strepto) [5]	TRF 43, 99 (BaPr) [6]	TRF 281, 284, 296 (Ccl4) [7]	TRF 75, 204, 220 (Ccl14a) [8]
S	n/d	1.69	9.41 [a]	64.39 [b]	1.52	n/d	0.20
C	n/d	9.59	3.79 [a]	27.25 [a]	3.68	0.70	n/d
E	n/d	2.05	8.95 [a]	37.17 [a]	3.09	0.87	n/d
L	n/d	1.73	22.99 [b]	35.97 [a]	1.35	1.23	0.60
P-Value		*>.10*	*0.01*	*0.01*	*>0.10*	*>.10*	*>.10*
SEM [9]		5.30	5.06	4.78	0.46	1.26	0.47

[1] Data in %, n/d, not detected; TRFs associated with [2]Enterobacteria, [3]*Clostridium* cluster I, [4]*Lactobacillus*, [5]*Streptococcus*, [6] *Bacteroides*, [7]*Clostridium* cluster IV and [8]*Clostridium* cluster XIVa; [9]SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P < 0.05$).

Table 6.13. Mean relative abundance (% of total bacteria) of selected major bacterial groups determined by qPCR in jejunal contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.

Treatment	Entero [2]	Ccl1 [3]	Lacto [4]	Strepto [5]	Bifido [6]	BaPr [7]	Ccl4 [8]	Ccl14a [9]
S	0.21 [b]	2.02 [(a)]	24.22	99.45 [b]	0.01 [ab]	2.48	0.03	0.01
C	0.12 [b]	1.35 [(a)]	16.23	27.33 [a]	0.001 [a]	3.02	0.02	0.01
E	0.07 [ab]	3.05 [(ab)]	22.60	83.03 [ab]	0.03 [b]	2.70	0.03	0.01
L	0.02 [a]	6.82 [(b)]	37.81	41.34 [ab]	0.03 [b]	2.51	0.04	0.01
P-Value	*0.002*	*0.91*	*>.10*	*0.02*	*0.05*	*>.10*	*>.10*	*>.10*
SEM [10]	0.04	3.13	12.73	25.14	0.02	1.72	0.03	0.02

Total bacteria $\log_{10}$ count/g of digesta 11.19 (S), 11.52 (C), 11.19 (E), 11.70 (L), $P > 0.10$, SEM 0.20; [2] Entero, Enterobacteria; [3] Ccl1, *Clostridium* cluster I species; [4] Lacto, *Lactobacillus* species; [5] Strepto, *Streptococcus* species; [6] Bifido, *Bifidobacterium* species; [7] BaPr, *Bacteroides* and *Prevotella* species; [8] Ccl4, *Clostridium* cluster IV species, [9] Ccl14a, *Clostridium* cluster XIVa species; [10] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different (P<0.05); [(a)-(b)] Values in same column with different superscripts are different ($P < 0.10$).

Table 6.14. Mean relative abundance (% of total bacteria) of inoculated bacteria determined by qPCR in jejuna mucosa collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.

Treatment	Cperf [2]	Sinfa [3]	Lmuco [4]
S	1.26	0.005	0.26
C	0.99	0.004	0.48
E	1.06	0.004	0.83
L	1.39	0.004	0.26
P-Value	*>.10*	*>.10*	*>.10*
SEM [5]	0.52	0.002	0.13

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of mucosa 9.47 (S), 9.44 (C), 9.44 (E), 9.55 (L), $P > 0.10$, SEM 0.32; [2] Cperf, *Clostridium perfringens*; [3] Sinfa, *Streptococcus infantarius*; [4] Lmuco, *Lactobacillus mucosae*; [5] SEM, standard error of the mean.

Table 6.15. Mean relative abundanc (% of total bacteria) of selected major bacterial groups determined by qPCR in jejunal mucosa collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups[1].

Treatment	Entero [2]	Ccl1 [3]	Lacto [4]	Strepto [5]	Bifido [6]	BaPr [7]	Ccl4 [8]	Ccl14a [9]
S	8.71 [ab]	12.94 [ab]	27.65	35.02 [b]	0.03	12.92	0.20 [(ab)]	0.24 [(b)]
C	2.11 [a]	22.57 [b]	28.46	17.56 [a]	0.01	17.25	0.06 [(ab)]	0.25 [(b)]
E	2.667[a]	20.56 [b]	33.34	22.80 [ab]	0.02	10.65	0.30 [(b)]	0.19 [(ab)]
L	17.23 [b]	4.40 [a]	27.57	28.26 [ab]	0.02	15.71	0.02 [(a)]	0.10 [(a)]
P-Value	*0.001*	*0.006*	*>.10*	*0.05*	*>.10*	*>.10*	*0.09*	*0.09*
SEM [10]	3.22	5.28	12.18	10.26	0.03	5.24	0.21	0.12

[1]total bacteria $\log_{10}$ count/g of mucosa 9.47 (S), 9.44 (C), 9.44 (E), 9.55 (L), $P > 0.10$, SEM 0.32; [2] Entero, Enterobacteria; [3] Ccl1, *Clostridium* cluster I species; [4] Lacto, *Lactobacillus* species; [5] Strepto, *Streptococcus* species; [6] Bifido, *Bifidobacterium* species; [7] BaPr, *Bacteroides* and *Prevotella* species; [8] Ccl4, *Clostridium* cluster IV species, [9] Ccl14a, *Clostridium* cluster XIVa species; [10] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P < 0.05$); [(a)-(b)] Values in same column with different superscripts are different ($P < 0.10$).

number / g of content (Table 6.14). Bacterial group specific analysis did not differ ($P > 0.10$) for *Lactobacillus, Bifidobacterium* and *Bacteroides / Prevotella* (Table 6.15). Enterobacteria were highest ($P = .001$) in treatment L, whereas all *Clostridium* clusters analyzed were lowest ($P < 0.09$) in treatment L (Table 6.15). *Streptococcus* was highest ($P < 0.05$) in treatment S. *Enterococcus* spp. abundance was not affected by treatment (data not shown). Species specific analysis for selected bacteria did not indicate significant treatment effects except for about 10 fold higher abundance of *L. reuteri* and *B. thermacidophilum* ($P < 0.01$) in treatment C animals vs. treatments E and L (data not shown). Overall, none of the microbial targets directly correlated with animal growth performance ($P > 0.10$).

6.4.6. Jejunal Histology

Treatment S animals, which had the poorest growth rate, showed the longest ($P = 0.001$) villi, deepest ($P = 0.06$) crypts, and the lowest ($P = 0.001$) villus height to crypt depth ratio (Table 6.16). Treatment C animals presented with intermediate villus and crypt characteristics (Table 6.16). Villus height and villus height to crypt depth ratio were correlated ($P < 0.05$, $R^2 = 0.74$ and 0.84, respectively) with pig growth rate. Sialylated, carboxylated and/or sulfated mucus secreting goblet cells were not altered by treatment. Interestingly, Treatment L animals showed markedly elevated ($P < 0.001$) counts of mixed mucin producing goblet cells on villi especially compared with treatment S where no mixed goblet cells were observed on villi (Figure 6.1).

6.4.7. Jejunal Gene Expression

For host immune related gene expression, no differences ($P > 0.10$) between treatments were observed for TRAF6 mediated cytokine expression of *IL-1β*, *IL-6*, *IL-8*, *IL-10*, as well as related death ligand *TNFα* and crypt cell proliferation related *PCNA* gene expression (data not

Table 6.16. Mean villus height, crypt depth, villus height to crypt depth ratio and number of goblet cells per villus and per crypt. [1]

Treatment	Villus				Crypt		Vh:Cd
	Height (μm)	Acidic goblet cells [2]	Mixed goblet cells [3]	Total goblet cells	Depth (μm)	Mixed goblet cells [3]	ratio
S	295.3 [b]	15.89	0.000 [a]	15.89	192.5 [(b)]	16.127	1.534 [a]
C	264.8 [ab]	15.17	0.083 [a]	15.25	139.9 [(ab)]	15.694	1.894 [ab]
E	245.5 [a]	14.82	0.313 [a]	15.13	84.3 [(a)]	16.000	2.917 [b]
L	257.8 [a]	14.28	2.610 [b]	16.88	102.0 [(ab)]	15.778	2.550 [b]
P-Value	*0.001*	*>.100*	*<0.001*	*>0.100*	*0.063*	*0.229*	*0.001*
SEM	25.2	1.27	0.114	1.30	19.561	2.369	0.286

[1] Average count per villus / crypt determined over 10 villi / crypts per animal, animal being experimental unit; [2]Acidic sialylated, carboxylated and/or sulfated mucus secreting goblet cells stained blue with PAS2.5/AB stain; [3] Mixed acidic and α-glycol-rich neutral mucus secreting goblet cells stained purple with PAS2.5/AB stain as defined in pigs by Law *et al.* (2007); [a-b] Values in same column with different superscripts are different ($P < 0.05$); [(a)-(b)] Values in same column with different superscripts are different ($P < 0.10$).

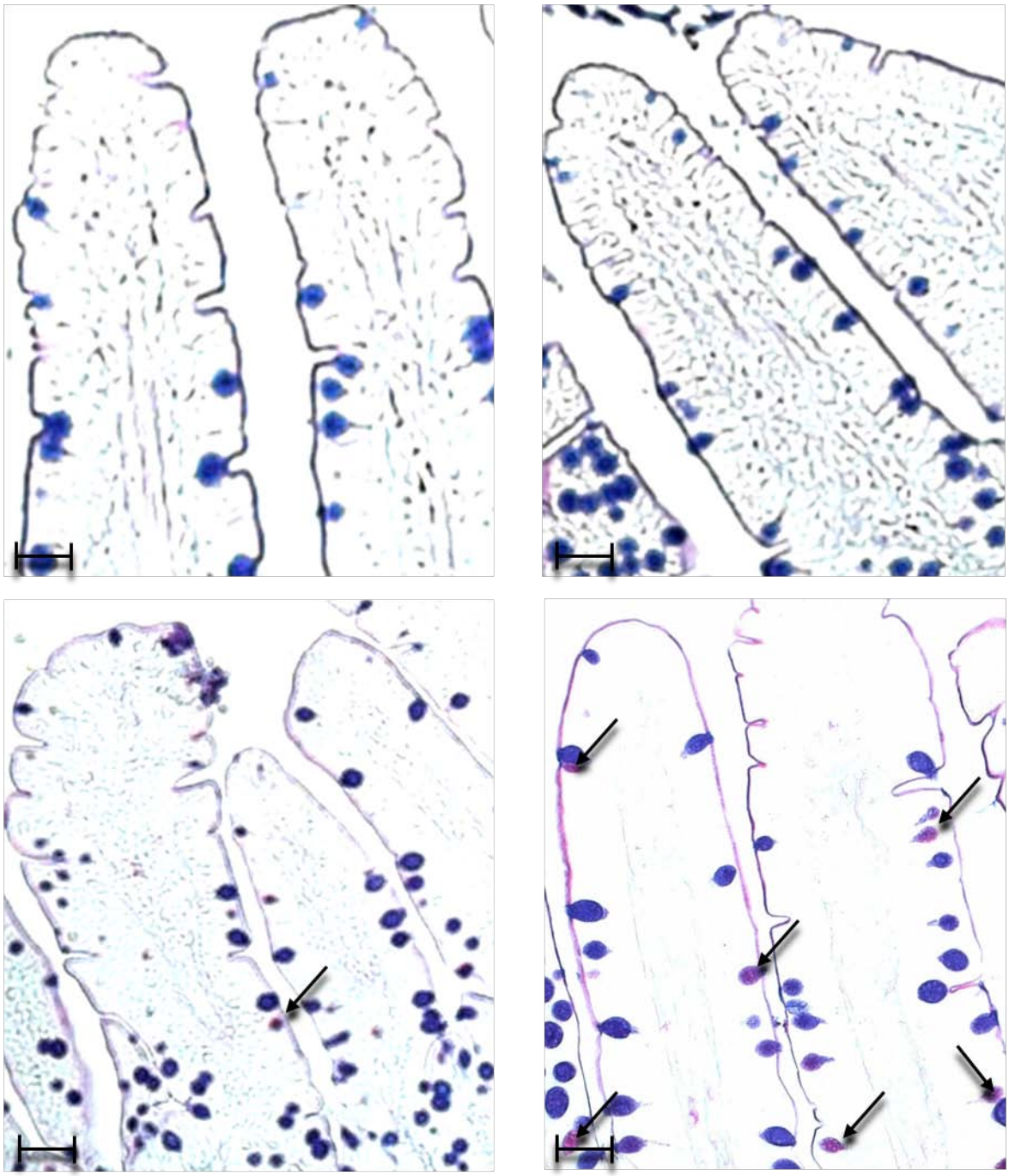

Figure 6.1. High contrast AB2.5/PAS and H&E stained villi from one representative pig jejunum from treatment S (top left), C (top right), E (bottom left) and treatment L (bottom right) showing goblet cells: blue goblet cells contain acidic sialylated, carboxylated and/or sulfated mucins, whereas a purple stained goblet cells contain a mixture of blue-stained acidic sialylated, carboxylated and/or sulfated mucin and red-stained α-glycol-rich neutral mucins (Fontaine and Meslin, 1994; Law *et al.*, 2007) and are indicated by arrows; bar indicates 25 µm.

shown). Furthermore, no differences ($P > 0.10$) were observed for TRAF3 mediated expression of *IRF3*, *IFNγ*, as well as for the related death ligand *FasL* (data not shown). Expression of immune status related genes with treatment effect ($P \leq 0.10$) are given in Table 6.17. Monoassociation with *Lactobacillus mucosae* early post-partum resulted in the highest expression ($P < 0.01$) of *TLR2* and *TLR4*, intermediate expression of *NFKB1* and *NFKB2* and highest expression of *NFKBIA*. Numerically lowest expression of *TLR2* and *TLR4* was observed in Treatment E pigs associated with the low expression of *NFKB1*, *NFKB2*, and *NFKBIA*. High expression of *TLR4* in treatment S was associated with the highest expression level of *NFKB1*. Treatment C animals showed low to intermediate immune related gene expression.

Mucus and digestion related gene expression is shown in Table 6.18. The most abundantly expressed mucin gene in jejunal tissue was *Muc13* followed by *Muc20*. *Muc1* was detected in very low abundance only (data not shown), making quantitation impossible. In treatment S pigs, *Muc13* expression was lowest ($P = 0.10$) and *Muc2* expression highest ($P < 0.05$). Treatment E animals showed highest ($P = 0.05$) expression of *Muc20* but lowest expression of *Muc2* ($P < 0.05$). Pigs from treatment L expressed most ($P = 0.10$) *Muc13*, but least ($P = 0.05$) *Muc20* whereas treatment C mucus expression was intermediate.

Regarding nutrient related gene expression, sodium-glucose linked transporter 1 (*SGLT1*) gene expression was not affected by treatment ($P > 0.10$, Table 6.18). From the remaining investigated gene targets, treatment L pigs showed the highest ($P \leq 0.07$) expression of brush border hydrolases and nutrient transporters whereas animals from treatment E demonstrated lowest expression of selected digestion and transporter genes with exception of the low affinity sodium-glucose linked transporter 3 (*SGLT3*). Animals from treatment S and C had lowest

Table 6.17. Mean bacterial sensing and immune status related gene expression in piglet jejunum intestinal tissue collected at 28 d of age for pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	*TLR2* [2]	*TLR4* [3]	*NFKB1* [4]	*NFKB2* [5]	*NFKBIA* [6]
S	16.44^{a}	136.9^{b}	$1069.4^{(b)}$	25.74^{ab}	205.1^{a}
C	15.59^{a}	78.1^{ab}	$861.6^{(ab)}$	16.92^{a}	233.3^{a}
E	8.17^{a}	31.0^{a}	$561.3^{(a)}$	21.57^{a}	145.1^{a}
L	33.85^{b}	140.3^{b}	$814.4^{(ab)}$	52.14^{b}	397.9^{b}
P-value	*<.001*	*0.003*	*0.067*	*0.012*	*<.001*
SEM [7]	7.56	60.9	293.9	19.27	83.1

[1] Data presented in gene copies per 1000 *GAPDH*, separation of means by Tukey HSD; [2] *TLR2*, toll-like receptor 2; [3] *TLR4*, toll-like receptor 4; [4] *NFKB1*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; [5] *NFKB2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; [6] *NFKBIA*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; [7] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P < 0.05$); [(a)-(b)] Values in same column with different superscripts are different ($P < 0.10$).

Table 6.18. Mean mucus and digestion related gene expression in the small intestine collected at 28 d of age for pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	*Muc2* [2]	*Muc13* [3]	*Muc20* [3]	*APN* [4]	*LPH* [5]	*PepT1* [6]	*SGLT1* [7]	*SGLT3* [8]
S	3150 [b]	1265 [(a)]	601 [ab]	8609 [ab]	6.65 [a]	18.29 [(a)]	3980	7.10 [a]
C	2386 [ab]	2241 [(ab)]	534 [ab]	7953 [ab]	4.36 [a]	35.98 [(ab)]	2474	3.57 [a]
E	1386 [a]	2174 [(ab)]	1005 [b]	4296 [a]	2.95 [a]	18.25 [(a)]	3019	8.73 [a]
L	2875 [ab]	2544 [(b)]	398 [a]	10650 [b]	13.49 [b]	38.77 [(b)]	4480	21.78 [b]
P-value	*0.03*	*0.10*	*0.05*	*0.01*	*0.03*	*0.07*	*0.15*	*0.02*
SEM [9]	1094	886	345	2967	5.79	3.70	679	2.52

[1] Data in gene copies per 1000 *GAPDH*, separation of means by Tukey HSD; [2] secreted mucin; [3] transmembrane mucin; [4] *APN*, Aminopeptidase N; [5] *LPH*, lactase-phlorizin hydrolase; [6] *PepT1*, peptide transporter 1; [7] *SGLT1*, sodium/glucose linked transporter 1; [8] *SGLT3*, low affinity sodium/glucose linked transporter 3; [9] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P < 0.05$); [(a)-(b)] Values in same column with different superscripts are different ($P < 0.10$).

($P < 0.05$) carbohydrate related gene expression only, treatment S animals were also lowest in peptide related gene expression ($P = 0.07$) in the small intestine.

6.5. Discussion

As previously demonstrated in Chapter 5 and by others (Thompson *et al.*, 2008; Davis *et al.*, 2007), differences in microbial environment early in life can result in significant differences in commensal bacteria composition and intestinal mucosal physiology in the post weaned animal. However, early postnatal intestinal microbial composition is difficult to consistently modify due to the many environmental factors influencing the succession profile (Thompson *et al.*, 2008; Chapter 4). Therefore, a gnotobiotic approach was adopted here in order to ensure controlled variation in the succession profile and association of postweaning observations with a specific species.

Inoculation bacteria were selected as abundant species identified in a previous neonatal succession profiling experiment (Petri *et al.*, 2010), representing a variety of microbial traits: Three different commensal Gram positive organisms were selected including, a non-toxin producing non-adherent bacterium (*S. infantarius*), a non-adherent toxin producing bacterium (*C. perfringens*), and a non-toxin producing but attaching bacterium (*L. mucosae*). In addition, a commensal non-attaching non-toxin producing Gram negative bacterium (*E. coli*) was used. Monoassociation of neonatal gnotobiotic pigs with one of the four organisms for four days was used to determine the effect of early microbial succession on post-weaning commensal microbiota composition.

Although our model was successful, one challenge of this approach was the relatively small number of animals that can be reared in isolators and the corresponding high level of biological

variation. Monoassociation with different bacteria for the first four days of life resulted in detectable changes in microbial profile at 28 days of age, one week following abrupt weaning. Furthermore, treatment differences in gut histology and host gene expression in pig jejunum were identified and correlated with variation in growth rate.

6.5.1. Treatment Effects On Host Growth Rate

Differences in microbial succession pattern throughout the first 4 days post-partum impacted piglet BW at 28 d of age and ADG from 7-28 d. Lowest ADG and final BW was found for pigs in *S. infantarius* monoassociated pigs compared to highest ADG and final BW in *E. coli* and *L. mucosae* monoassociated pigs. Increased growth rate in *L. mucosae* monoassociated pigs was consistent with our previous observations of the effect of *L. fermentum* monoassociation on small intestinal morphology and gene expression (Shirkey *et al.* 2006; Willing and Van Kessel, 2007) in the pig and with the growth performance of young pigs supplemented with probiotic *Lactobacillus* spp. (Davis *et al.* 2007; Konstantinov *et al.* 2008).

Growth performance improvement in *E. coli* monoassociated pigs was unexpected since commensal *E. coli* strains have not been previously shown to enhance pig growth rate. Liu *et al.* (2008) determined that increased fecal *E. coli* counts coincided with diarrhea incidence and severity, reducing animal growth performance post weaning. Interestingly, *Lactobacillus* spp. and *E. coli* have very distinct biochemical features, nutrient requirements and metabolites (Bergey and Holt, 1994), despite their common positive influence on growth performance in the present study.

Clostridium perfringens was the only toxin producing inoculant in this experiment, a known pathogen of neonatal pigs (Songer and Taylor, 2006) and therefore was anticipated to negatively influence growth. However, *C. perfringens* showed an intermediate effect on growth and indeed

was well tolerated even in monoassociation. This finding is highly supportive of the hypothesis that pathogenicity of *C. perfringens* is highly strain specific (Baker *et al.*, 2010).

Streptococcus infantarius monoassociation was associated with lowest animal growth performance. This was surprising because the *Streptococcus* genus include a number of probiotic species (Huaynate *et al.*, 2006) and is most closely related to the *Lactobacillus* genus. Similar to research shown here, Schmidt *et al.* (2011) examined the effect of bacterial succession pattern in pigs by comparing conventionally-reared and isolator-reared piglets which had been removed from the sow at 1 day of age. In agreement with our findings, altering the succession profile affected postweaning growth rate, however, none of the bacterial species affected by the preweaning rearing environment could be correlated with growth performance parameters. The present study suggests that the period of influence of succession profile on subsequent growth performance is very early in the postnatal period and establishes a relationship between specific species and host responses.

6.5.2 Microbial Ecology

Although TRFLP banding patterns showed no evidence of clustering by treatment, TRF abundance and qPCR enumeration of selected taxa in jejunal contents and mucosa indicated consistent treatment effects particularly with respect to the relative abundance of *Streptococcus* and *Lactobacillus* spp. This finding is remarkable in that the treatment groups differed only in the single species of bacteria that was permitted to colonize the intestine during the first four days of life. This was consistent with Schmidt *et al.* (2011) who showed clustering of the small intestine mucosa-adherent microbiota from outdoor derived pigs up to weaning on day 28 and further supported by a study in the human intestine, where one week of antibiotic exposure resulted in resistance of *Bacteroides* spp. two years later (Löfmark *et al.*, 2006).

Based on these findings, we first asked whether the abundance of the monoassociated bacterial species was increased or decreased in contents and mucosa after weaning. Since the relative abundances of the monoassociated species were very low postweaning this likely contributed to highly variable values among pigs and the lack of statistical differences. Nevertheless the data does speak against the “first come first to occupy” hypothesis where the first to occupy a niche may be advantaged in retaining the niche.

Secondly, we asked whether the genus of the monoassociated bacterium was advantaged or disadvantaged in the post weaning gut. In this case, monoassociation with *S. infantarius* increased the relative abundance of *Streptococcus* species in the jejunal mucosa and contents. However, no other association between the monoassociated genus, and its relative abundance post weaning could be made. Monoassociation with *L. mucosae* may have advantaged *Lactobacillus* spp. in jejunal contents but no relationship was obvious in jejunal mucosae. Enterobacteria were observed at low abundance in jejunal contents and intermediate abundance in jejuna mucosa of *E. coli* monoassociated pigs supporting a negative relationship. *Clostridium* cluster 1 bacteria were lowest in jejunal contents but highest in jejuna mucosa of *C. perfringens* monoassociated pigs. Abundance of the other Clostridial clusters did not appear to follow any pattern.

A number of mechanisms could potentially mediate the effect of early colonization on postweaning microbial profile. The effect could be either ‘direct’ affecting microbial colonization of the monoassociated genus and/or species, or may be an ‘indirect’ via succession differences after the monoassociation phase. As an example for a direct mechanism, *B. thetaiotaomicron* is capable of affecting the host mucin composition to serve as a substrate and to give itself a competitive advantage over other mucosa colonizing organisms (Hooper *et al.*,

2001). It could be speculated that *S. infantarius* and *L. mucosae* may have impacted mucin post translational processing in a similar fashion to give the genus the competitive advantage observed.

Alternatively, via the indirect mechanism, exposure by monoassociation may have activated host tolerance or immunity to the monoassociated organism, affecting subsequent colonization patterns. Gerdts *et al.* (2002) demonstrated that the adaptive immune system is already active and can be affected before birth using one time oral *in utero* vaccination in lambs. Host tolerance to the respective pathogen was determined at birth and three months post-partum, demonstrating the ability of the adaptive immune system to be affected even before birth with a lasting effect. Furthermore, since commensal microbiota can strongly influence mucosal IgA secretion and binding affinity (Macpherson and Slack, 2007), which in turn is selectively affected by commensal microbiota in the gut (Hansen *et al.*, 2010), inoculant-mediated effects on secreted IgA specificity could account for postweaning colonization differences. Differences in *TLR4* expression as determined in this study might be responsible for up regulation of polymeric immunoglobulin receptor, the major IgA transporter causing increased IgA secretion into the gut mucosa, as it was determined by Bruno *et al.* (2010) in the human colon. A mix of both mechanisms could potentially explain how alterations in microbial ecology up to 4 days of age could alter commensal microbiota post weaning.

6.5.3. Small Intestinal Mucosa

The ratio of villus height to crypt depth (Vd:Cd) correlated with growth performance. The largest ratio was observed for *E. coli* and *L. mucosae* monoassociated pigs and the smallest values for pigs monoassociated with *S. infantarius*. Malik (2009) and Gebert *et al.* (2008) also noted a positive correlation between growth rate and Vh:Cd ratio in pigs. In this context, the ratio

is often associated with epithelial turnover rate and thus maturity. However, we did not observe differences in expression of genes associated with epithelial turnover (*PCNA*) in the present experiment.

Since differences in *TLR2* and *TLR4* gene expression were significantly affected by treatment, but neither altered inflammatory cytokine gene expression nor *IRF3* and interferon related gene expression, the NFκB signaling pathway (*NFKB1*, *NFKBIA* and *NFKB2*) became focus of the immune related analysis here. Pig monoassociated with *S. infantarius* were slowest growing associated with high expression of *TLR4* and *NFKB1*. Although intretation based on gene expression alone must be cautioned, the pattern coulsdd suggest heightened surveillance of and responsiveness to pro-inflammatory signals. In contrast, *L. mucosae* monoassociated pigs had the highest growth rate and high expression of both *TLR2* and *TLR4*. For these pigs, intermediate expression of *NFKBI* and *NFKB2* were observed but in this case expression of the inhibiting IκBα precursor *NFKBIA* was also elevated, possibly normalizing responsiveness to inflammatory stimuli (Hay *et al.*, 1999). *Escherichia coli* monoassociated pigs had similar growth rate to *L. mucosae* associated pigs but in this case, *TLR2*, *TLR4* and NFκB transcription factors were all in lowest abundance among the treatment groups. Comparing *E. coli* and *L. mucosae* inoculated animals, both of which showed the best growth performance, it seems microbial sensing and overall immune response in the gut was reduced in *E. coli* treated pigs potentially due to overall consistently low toll-like receptor and mediator gene expression. This host response was somewhat similar to mice exposed to *E. coli* and stimulated with dextran sodium sulfate which did not develop colitis via TLR signaling down regulation (Grabig *et al.*, 2005). However, to the best of the author's knowledge, similar research has not been done in piglets.

Goblet cell staining for mucins was found to be similar to findings of Deplancke and Gaskins (2001) showing mostly acidic mucus in the pig gut post weaning. However, Uni *et al.* (2003) related the appearance of neutral and mixed mucus secreting goblet cells in the small intestine to a greater degree of host maturity based on increased activity of the adaptive immune system with age (Cebra, 1999). The relative expression of *Muc2* in this study was found to be comparable in abundance to results from pig ileum of gnotobiotic pigs (Malik, 2009). As with *TLR2* and *TLR4*, *Muc2* expression was highest in *S. infantarius* and lowest in *E. coli* inoculated animals. The *Muc2* gene encodes a secreted mucin which is mostly described as having a 'washing out' effect on microorganisms as a defense mechanism (Pearson and Brownlee, 2005).

It is possible that increased *Muc2* expression in *S. infantarius* inoculated pigs might be linked to the increased sensitivity to Gram-negative organisms via the TLR pathway (Figure 6.3; Kawai and Akira, 2007; Deplancke and Gaskins, 2001). Increased *Muc2* gene expression has generally been associated with improved gut barrier function (Malik, 2009, Smith *et al.*, 2011). In contrast to *S. infantarius* inoculated animals, and consistent with *TRL2* and *TLR4* expression patterns, *E. coli* inoculated pigs showed the lowest *Muc2* expression, but highest expression of some transmembrane mucins (*Muc20*).

Interestingly, *L. mucosae* inoculated piglets, which had significantly increased levels of *TLR2*, *TLR4* and the *NFKBIA* inhibitor only had moderate levels of *Muc2* expression and the lowest levels of *Muc20* for all treatments. This further indicates that *Muc2* as well as *Muc20* gene expression are potentially linked to toll-like receptor sensing of gut microbiota. Not surprisingly, given the contrasting mucin expression profiles in pigs from the two fastest growing treatment groups, mucin gene expression did not correlate with growth performance. It would be

interesting to investigate whether performance responses would diverge in these two treatment groups in response to enteric pathogen challenge.

Martin II *et al.* (2003) determined that artificially induced immune activity, even though it did not lead to inflammation, resulted in a significant increase of resting metabolic rate. Based on these findings, it could be assumed that the growth rate improvement in *E. coli* inoculated pigs could be due to a 'muted' sensing of immunostimulatory bacterial products. Reduced sensing of bacterial products was not apparent as a mechanism of action for growth performance improvement in the *L. mucosae* treatment group, however, some other mechanism appears active to limit inflammation through expression of *NFKBIA*. Nevertheless, an increase in energy expenditure for resting metabolic rate in *S. infantarius* inoculated animals could be an explanation for the reduced growth rate of treatment S pigs.

Few publications have addressed digestion related gene expression in livestock (Malik, 2009) yet it is commonly believed that differences in growth performance are related to energy efficiency of digestion and the expression of digestion related genes (Moran *et al.*, 2010). Several digestion related genes were analyzed in this experiment and all except *SGLT1* were found to be significantly higher in *L. mucosae* inoculated animals, consistent with an increased villus height to crypt depth ratio. Both, *APN* and *LPH* have been shown to be significantly reduced in neonatal pigs when milk replacer was fed instead of porcine colostrum, indicating other early postnatal environmental factors can modify subsequent mucosal hydrolase expression (Jensen *et al.*, 2001). This might be applicable to *E. coli* inoculated animals which in spite of their high mature villus height to crypt depth ratio had lowest *APN* and *LPH* gene expression. Similarly, Willing and Van Kessel (2009) determined that *APN* and *LPH* gene expression was

impacted by microbial colonization in a gnotobiotic pig study, although brush border enzyme activity was not directly correlated to the respective gene expression.

Significant differences were detected in expression of the putative glucose sensing (Díez-Sampedro *et al.*, 2003) and passive glucose transporter protein *SGLT3* (Díez-Sampedro *et al.*, 2001), with *L. mucosae* treatment animals having more than twice the expression levels when compared to other treatments. It was recently determined that *SGLT3* was less important as a glucose transporter in the brush border membrane, but crucial as glucose sensor (Raybould, 2008), indicating a potential for increased ability of *L. mucosae* to detect dietary glucose. No differences in *SGLT1* levels in the brush border due to different microbial inoculants were detected. Moran *et al.* (2010) described differences in *SGLT1* gene expression due to feeding of sweeteners to weanling pigs, which correlated to an increase in feed intake in piglets post weaning. However, due to co-housing, it was not possible to determine treatment specific feed intake in the current study.

Lastly, *L. mucosae* inoculated animals showed significantly higher expression of gene *PepT1* compared to *E. coli* and *S. infantarius* inoculated pigs. Although ShiGeng *et al.* (2009) described differences in *PepT1* expression due to gut location, age of the animal and breed, there are no studies connecting gut microbial ecology and *PepT1* gene expression in pigs. However, Shu *et al.* (2002) showed that *PepT1* expression in the rat jejunum was negatively impacted by LPS injection, which somewhat coincided with overall lowest *PepT1* gene expression in *E. coli* monoassociated pigs in the current study. Similar to TLR and mucin gene expression patterns, the two fastest growing treatment groups, demonstrated the most divergent patterns in nutrition related gene expression. *Lactobacillus mucosae* animals had consistently highest nutrition related gene expression, which might be one mechanism of improved animal growth rate up to day 28.

In contrast, high growth rate in the *E. coli* inoculated group was not likely due to improvements in carbohydrate or peptide sensing, digestion or uptake.

6.6. Conclusions

Monoassociation of pigs with different bacterial species in the early postnatal period affected animal growth rate, intestinal microbial ecology, nutrient assimilation and barrier function-related gene expression post weaning. Although marked changes in postweaning microbial profiles were not observed, abundance of specific taxonomic groups was altered. Depending on the monoassociated genus, early colonization appeared to promote or inhibit subsequent colonization. Highest growth rate was observed in pigs monoassociated with *L. mucosae* and *E. coli*, two taxonomically and biochemically diverse organisms. Despite a similar growth rate, these diverse organisms were associated with opposing postweaning gene expression profiles. Whereas *L. mucosae* associated pigs demonstrated a relatively high abundance of transcripts for microbial recognition receptors, inflammatory transcription factors, mucin genes and nutrient assimilation genes, *E. coli* monoassociated pigs demonstrated low abundance of these transcripts. We conclude that bacterial colonization patterns experienced within the first 4 days of life in the pig influence postweaning microbial ecology and mucosal physiology in a bacterial species specific manner.

7.0 MICROBIAL PROGRAMMING IN PIGS: EFFECTS OF CONTROLLED SUCCESSION PATTERN ON LOWER GUT MICROBIAL ECOLOGY AND HOST RESPONSE POST WEANING

7.1. Abstract

Early postnatal exposure to environmental microbiota has a significant impact on gut microbial composition and the nature of host-microbiota interactions which can potentially impact animal health and productivity. To determine how early postnatal distal intestinal bacterial succession affects microbial composition and intestinal physiology post-weaning, 24 piglets, derived by caesarian section, were mono-associated for the first 4 d of age with either *L. mucosae* (L), *S. infantarius* (S), *C. perfringens* (C) or *E. coli* (E). Animals were weaned on 21 d of age to a non-medicated pig starter diet, and then killed on 28 d of age to permit collection of the colonic digesta and mucosa for microbial analysis, and colonic tissue for histological and gene expression analysis. In colon digesta, volatile fatty acid analysis indicated an increase ($P < 0.05$) in butyrate concentration in *L. mucosae* inoculated animals compared with *S. infantarius* inoculated animals. However, no difference in microbial composition due to treatment was observed in colon digesta. In colonic mucosa, monoassociation with *C. perfringens* and *L. mucosae* resulted in a decrease ($P \leq 0.05$) in *Clostridium* and *Lactobacillus* spp., respectively. A significant decrease ($P < 0.001$) in abundance of *L. mucosae* was observed in the *L. mucosae* monoassociated pig but a corresponding decrease in the inoculating species was not observed for any other treatment. Mucosal effects included an increase in the number of goblet cells ($P < 0.05$) and *Muc2* gene expression ($P < 0.01$) in pigs inoculated with *S. infantarius* and *L. mucosae*

compared with *E. coli*, whereas treatment *S. infantarius* inoculated animals had the deepest crypts ($P < 0.01$). Although cytokine gene expression was not altered due to treatment ($P > 0.10$), differences in toll-like receptor dependent signaling were detected, where *E. coli* inoculated animals had consistently the lowest ($P < 0.05$) TLR gene expression comparing to *S. infantarius* inoculated pigs. This research clearly shows that controlled early microbial succession in neonatal pigs alters post-weaning commensal microbiota composition, gut histology and host gene expression in the large intestine.

7.2. Introduction

Our understanding of the importance of microbial colonization on the intestine of mammals such as pigs and humans is constantly developing and the roles of commensal bacteria in host nutrition, pathogen exclusion, development of immunity, and contribution to diseases such as cancer and obesity is becoming increasingly clear (Turnbaugh *et al.* 2006; Kelly *et al.*, 2007; Thompson *et al.* 2008). Colonization of the initially sterile intestinal tract proceeds rapidly after birth by bacteria both of maternal and environmental origin and succeeds to a more complex, mature ecosystem (Swords *et al.*, 1993; Conway 1996; Petri *et al.*, 2010). This leads to the concept that the best opportunity to modify colonization patterns and adult microbiota and therefore host health, is early in life (Kelly *et al.*, 2007). Chen *et al.* (2005) was able to demonstrate that supplementation of 14 day old mice with a *Lactobacillus* sp. probiotic bacterium was able to prevent colitis in mice when challenged with *C. rodentium* around 7 weeks of age. Mulder *et al.* (2009) and Schmitt *et al.* (2011) demonstrated that neonatal hygiene was crucial in microbiota succession and affected long term host gene expression in pigs. In human infants, Harmsen *et al.* (2000) and Apajalahti *et al.* (2001) determined that feces from breast fed infants was higher in *Bifidobacterium* and lower in *Bacteroides* spp. compared with formula fed

infants, factor which could contribute to the lower incidence of allergy in breast-fed infants later in life (Kirjavainen *et al.*, 2002). Thompson *et al.* (2008) also showed that co-housing was an important factor in determining microbial colonization in pigs for the first 42 days of life.

Although the above studies are indicative of a link between early succession of gut microbiota and subsequent community structure and health status of the host, a clear association between succession patterns for specific bacterial species and altered host response has not been established. We therefore utilized a gnotobiotic rearing system to precisely establish different early postnatal succession patterns. We hypothesized that monoassociation in early postnatal period in pigs alters post-weaning commensal microbiota composition and mucosal physiology in the distal gut in a manner dependent on the monoassociated species. The effect of early postnatal monoassociation on postweaning microbial profile and mucosal physiology in upper intestine was reported previously.

7.3. Materials and Methods

7.3.1. Experimental Design and Sample Collection

The experimental design and animals used are reported in Chapter 6, section 6.3.1. Briefly, twenty-four piglets were derived by Cesarean-section from 2 sows (WCVM, University of Saskatchewan, Saskatoon, SK, Canada). Before respiration was initiated, pigs were transferred to a common sterile HEPA-filtered isolator environment and orally administered by syringe to the oral cavity a mixture of 1.1 L sterile water, 250 g irradiated (5Mrad) spray dried bovine colostrum (HeadStart®, Saskatoon Colostrum Company Ltd, Saskatoon, SK, Canada), 50 g irradiated porcine animal blood plasma (AP 920®, APC Nutrition Ltd., Verchères, QC, Canada) and 80 mL irradiated medium-chain triglyceride oil (MCT Maxx^{TM}, PVL Nutrients Ltd, Mississauga, ON, Canada) in 4 doses of 3 mL each. Within 6 hours, pigs transferred from the

common isolator to one of four gnotobiotic isolators (n=6 per isolator) balanced for gender and litter of origin until 7 d of age. A 2:1 (v/v) mixture of iron fortified infant formula (Similac ® Advance ®, Abott Laboratories Limited, Saint-Laurent, QC, Canada) and water supplemented with 1% AP 920® was fed by bottle until 2 days of age. Thereafter, the mixture was adjusted to feeding *ad libtum* by trough using a 1.5:1 (v/v) mixture of formula to water. Pigs within each isolator were inoculated with 2 x 10^8 cfu of either *L. mucosae* (L), *S. infantarius* (S), *C. perfringens* (C) or *E. coli* (E) within 12 hours post-partum by mixing the appropriate bacterium with the milk replacer formula. Fecal swabs were collected at 3 and 4 d of age to confirm monoassociation with selected bacteria (sections 6.3.3 and 6.4.1). All pigs were normalized by inoculation with approximately 150 mg sow feces inoculant as described in chapter5, at 4 d of age, then adjusted to a commercial milk replacer formula (Wet Nurse®, Prairie Micro-Tech Inc., Regina, SK, Canada) and transferred to raised floor pens at 7 d of age (2 pigs per isolator were placed in one of 3 pens; 8 pigs per pen). Pigs were weaned to a commercial un-medicated pig starter (Whole Earth Pig Start®, Federated Co-Operatives Limited, Saskatoon, SK, Canada) at 20 d of age.

Animals were euthanized at 28 d of age to permit sample collection. Two 5 cm segments of mid colon were dissected and snap frozen in liquid nitrogen to permit analysis of mucosa adherent microbiota and host gene expression, and an additional 1 cm segment was fixed in 10% buffered formalin for histological analysis. All digesta were collected colon homogenized and sub-sampled for volatile fatty and commensal microbiota analysis. Digesta and mucosa samples were stored at -80 °C until processing for analysis. The study protocol (AUP number 20070073) was reviewed using guidelines established by the Canadian Council on Animal Care (Olfert *et al.*, 1993) and approved by the University of Saskatchewan Animal Research Ethics Board.

7.3.2. Molecular Microbial Analysis

Mucosa was harvested from thawed tissue segments after removal of contents and scrapping of mucosa with a microscope slide. Genomic DNA was extracted from digesta and mucosa as previously described (section 6.3.4; Dumonceaux *et al.*, 2006).

7.3.2.1. Terminal-restriction fragment length polymorphism (TRFLP) of microbiota groups and species

Terminal-restriction fragment length polymorphism (TRFLP) analysis was performed using the procedure and analytical methods of Fernando *et al.* (2010) with modifications as described in section 6.3.5.

7.3.2.2. Quantitative polymerase chain reaction (qPCR) of microbiota groups and species

Quantitative PCR (qPCR) was performed using primers and annealing temperatures given in Table 4.1. As previously reported in section 6.3.6, all reactions contained 0.02 µL each of 25 µM forward and reverse primers, 7.96 µL ddH_2O, 2 µL of template cDNA and 10 µL SsoFast^{TM} EvaGreen® Supermix (BioRad) and a two-step cycling reaction was used. Enumeration of bacteria groups and species was conducted using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, Canada). Standard curves were generated using pooled extracted DNA as template and primers described in Table 4.1. Amplicons were spectrophotometrically ($O.D._{260nm}$) and standards were converted to copy numbers using the formula:

Number of copies = [DNA (pg) * $6.022x10^{23}$ (copies per mole)] / [fragment length (bp) * $1x10^{12}$ (pg / g) * 650 (g / mole of bp)]

All amplifications were followed by melt curve analysis (BioRad CFX manager software, version 1.6.541.1028) to ensure single product amplification. Duplicate threshold cycles (Ct)

were averaged and mean values with greater than ± 0.50 standard deviations were reanalyzed. Acceptable reaction efficiency (E) was set for the range 0.90 - 1.10, and standard curve R^2 values to ≥ 0.97. Results were expressed in no. of gene copies per g of content or per g of mucosa using formula below:

Number of copies / g of content or mucosa = [Vol. after DNA extraction (µL) / IW content (g)] x [Dilution Vol. (µL) / Vol. aliquot in dilution (µL)] x [Number of copies / aliquot for qPCR reaction (µL)]

7.3.3. Volatile Fatty Acid (VFA) Analysis

Digesta samples were placed on ice and 50 mg weighed into a 2 mL micro-centrifuge vial containing 200 µL of 25% metaphosphoric acid. After vortexing for 2 min, samples were centrifuged at 10,000 ×g for 15 min (Microfuge ® 18, Beckman Coulter™, Palo Alto, CA, USA).The supernatant (120 µL) was transferred to a new 2 mL micro-centrifuge vial containing 500 µL trimethlyacetic acid and 880 µL acetonitrile. Samples were then vortexed for 2 min and centrifuged again at 14,000 rpm for 15 min. Supernatant (1 mL) was filtered through a microfiber syringe filter (0.45 µm diameter, Whatman ® GD/X, Sigma-Aldrich, St. Louis, MO, USA) into two gas-chromatograph vials. A standard curve was established ranging from 1.0 to 0.1 mM concentration and was prepared from a stock solution (Nu-Chek Prep Inc., Elysian, MN, USA) for acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic acid. Samples were analyzed using Agilent 6890 series GC system with Agilent 7683 series injector and Agilent Technologies High Performance GC Capillary Column (30.0 m x 320 µm x 0.25 µm; Agilent, Wilmington, DE, USA). Data was expressed in mmol volatile fatty acids per g of digesta. Concentrations of heptanoic acid were close to or below detection level and are not shown.

7.3.4. Histological Analysis

Using the previously described methodology from section 6.3.7, cross sections of the central colon were stained and analysis was performed looking at crypt depth in Gill's hematoxylin and eosin (H&E)-stained cross sections. Counts of carboxylated, sialated and/or sulfated acidic mucin secreting goblet cells per crypt in 10-fold replication per animal were determined via slides stained with 1% Alcian blue pH 2.5 staining with periodic acid and Schiff base reaction (AB2.5/PAS) overlaid with H&E (Law *et al.*, 2007).

7.3.5. Gene Expression Analysis In Colon Tissue

The 5 cm segment of central colonic tissue was pulverized with a clean, disinfected, RNAse treated (RNaseZap® Wipes, Applied Biosystems, Streetsville, ON, Canada) and liquid nitrogen cooled mortar and pestle. Ribonucleic acid was extracted under liquid nitrogen from an approximate 35 mg homogenized subsample using the RNeasy® Mini Kit (Qiagen, Montreal, QC, Canada). Resulting RNA was quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and processed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using a CFX96 real-time PCR detection system with a C1000 thermal cycler (BioRad, Guénette, QC, Canada) using previously described reaction and cycling conditions from section 6.3.8 (Tables 6.2, 6.4 and 6.5). One additional primer set for glucagon-like peptide 2 receptor (*GLP-2R*; Petersen *et al.*, 2001) was validated and utilized in this study. Primer sequences (5'-3') for *GLP-2R* were forward ACCTTGCAGCTGATGTACAC, reverse GTGTTCTCCAGGTGTGCACG with an annealing temperature of 55 °C and an amplicon size of 461 bp. Primer specificity was performed for all primers using gel purification, extraction and Sanger sequencing of the PCR product. For gene expression primers, sequence results were blasted using NCBI nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with option Refseq mRNA referring to *sus scrofa*

database (taxid:9823). Sanger sequencing result of the *GLP-2R* PCR product was 99% homologue to NCBI accession# NM_001246266.1 annotated to *GLP-2R* in the pig with no other hits. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-actin (*ACTB*) were quantified and neither gene showed treatment related significance in expression. However, *ACTB* demonstrated high animal to animal variation and therefore data was normalized to *GAPDH* only.

7.3.6. Statistical Analysis

Prior to statistical analysis, qPCR data was $\log_{10}$ transformed (total bacteria counts), and TRFLP relative peak area and qPCR relative comparison to total bacteria was normalized using the formula:

Normalized relative value = [arc sin $\sqrt{}$ (relative value)] (Fernando *et al.,* 2010)

Preliminary analysis was performed in a 2x2x4 factorial ANOVA with factors gender, litter of origin and treatment. Since factors gender, litter of origin and their corresponding interactions were not significant, the respective factors were removed from the model. Data presented were analyzed as one-way ANOVA using Proc Mixed procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA) and Tukey HSD for treatment mean separation. Pairwise Pearson correlation analysis was performed using Proc Corr procedure of SAS. For all tests, significance was declared at $\alpha \leq 0.05$, trends were indicated for $0.10 \geq \alpha > 0.05$.

For banding pattern analysis, TRF profiles for each sample were imported into Bionumerics software version 5.1 (Applied Maths, TX, USA). Band based fingerprint UPGMA cluster analysis was performed using Dice and Jaccard option with 1% position tolerance. Bacterial community diversity was determined using Shannon-Weiner index (Shannon and Weaver, 1949) based on relative peak areas of MspI TRFLP profiles of individual samples.

7.4. Results

7.4.1. Volatile Fatty Acid (VFA) Analysis

Total VFA concentration in colonic digesta ranged from 103 μmol/g of contents for treatment E to 92 μmol/g of content for treatments S and C, with treatment L being intermediate with at 100 μmol/g of content ($P = 0.08$, Table 7.1). Butyrate concentration was higher ($P < 0.05$) in colon contents of animals in treatment L compared to treatment S and was positively correlated ($P < 0.05$, $R^2 = 0.68$) with the previously reported animal growth rate (section 6.4.1).

7.4.2. Microbiota in Colon Contents

Assessment of microbial ecology via TRFLP banding patterns revealed no clustering by treatment group ($P > 0.10$), nor was the Shannon diversity index different ($P > 0.10$) among treatments on day 28. There were also no significant differences ($P > 0.10$) in the relative abundance of TRFs 28, 460, 494 and 520 which represent, but are not unique to the monoassociated bacterial species *L. mucosae, S. infantarius, E. coli* and *C. perfringens*, respectively. Similarly, qPCR enumeration of the monoassociated species in colon content, indicated no effect of treatment ($P > 0.10$; Table. 7.2) although high individual variability was observed. The abundance of monoassociated species within the colon was below 0.1% of total bacteria for *C. perfringens* and *S. infantarius*, and below 1% of total bacteria for *L. mucosae*. Terminal restriction fragments associated with enterobacteria and *Bifidobacterium* were not detected via TRFLP analysis (Table 7.3) of colon digesta, but could be enumerated via qPCR (Table 7.4). The relative abundance of *Bacteroides / Prevotella* and *Clostridium* cluster I, IV and XIVa whether expressed as percent of TRF peak area or determined by qPCR. No treatment

Table 7.1. Mean volatile fatty acid (VFA) concentration (µmol/g) in colon digesta. [1]

Treatment	Acetic Acid	Propionic Acid	Butyric Acid	Valeric Acid	Isobutyric Acid	Isovaleric Acid	Total VFA [2]
S	50.19	29.48	9.30 [a]	1.45	0.70	0.61	91.73 [(a)]
C	51.46	27.86	11.25 [ab]	1.17	0.52	0.42	92.48 [(a)]
E	54.75	31.79	14.01 [ab]	1.69	0.43	0.46	103.13 [(b)]
L	52.01	29.14	16.20 [b]	1.38	0.64	0.57	99.94 [(ab)]
P-value	*0.323*	*0.302*	*0.049*	*0.227*	*0.133*	*0.469*	*0.080*
SEM [3]	4.26	3.62	3.90	0.44	0.20	0.23	7.12

[1] Data in µmol VFA per g sample fresh matter, means separated by Tukey HSD; [2] Calculated as sum of individual VFAs; [3] SEM, standard error of the mean; [a-b]Values in same column with different superscripts are different ($P<0.05$); [(a)-(b)]Values in same column with different superscripts are different ($P<0.10$).

Table 7.2. Mean relative abundance (% of total bacteria) of inoculated bacteria determined by qPCR in colon contents collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	Cperf [2]	Sinfa [3]	Lmuco [4]
S	0.054	0.039	0.521
C	0.049	0.050	0.312
E	0.032	0.027	0.704
L	0.081	0.043	0.325
P-Value	*>.100*	*>.100*	*>.100*
SEM [5]	0.016	0.018	0.113

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of digesta 12.27 (S), 12.24 (C), 12.72 (E), 12.18 (L), $P > 0.10$, SEM 0.84; [2] Cperf, *Clostridium perfringens*; [3] Sinfa, *Streptococcus infantarius*; [4] Lmuco, *Lactobacillus mucosae*; [5] SEM, standard error of the mean.

Table 7.3. Mean normalized peak area as terminal restriction fragments (TRFs) assigned to selected major taxonomic groups in colon digesta of pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	TRF 194, 494 (Entero) [2]	TRF 516, 520 (Ccl1) [3]	TRF 28, 152, 155, 178, 188, 566 (Lacto) [4]	TRF 48, 460, 553 (Strepto) [5]	TRF 43, 99 (BaPr) [6]	TRF 281, 284, 296 (Ccl4) [7]	TRF 75, 204, 220 (Ccl14a) [8]
S	n/d	4.55	0.50	29.65 [a]	7.19	6.76	5.77
C	n/d	5.75	0.37	54.85 [b]	6.52	6.91	7.77
E	n/d	6.88	0.83	47.65 [b]	5.56	9.90	4.05
L	n/d	5.20	0.21	50.01 [b]	7.08	3.78	5.57
P-Value		*>.10*	*>.10*	*0.03*	*>0.10*	*>.10*	*>.10*
SEM [9]		1.92	0.12	3.30	1.19	4.91	2.00

[1] Data in % of total peak area, separation of means by Tukey HSD, n/d, not detected; TRFs associated with [2] Enterobacteria, [3] *Clostridium* cluster I, [4] *Lactobacillus*, [5] *Streptococcus*, [6] *Bacteroides*, [7] *Clostridium* cluster IV and [8] *Clostridium* cluster XIVa; [9] SEM, standard error of the mean; [a-b]Values in same column with different superscripts are different ($P<0.05$).

Table 7.4. Mean relative abundance (% of total bacteria) for selected major taxonomic groups in colon digesta of pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	Entero [2]	Ccl1 [3]	Lacto [4]	Strepto [5]	Bifido [6]	BaPr [7]	Ccl4 [8]	Ccl14a [9]
S	0.66	3.22	7.09	15.85	0.0021 [(ab)]	9.58	7.01	1.73
C	0.26	2.30	16.62	26.69	0.0019 [(ab)]	9.91	7.39	1.54
E	0.57	3.35	9.75	41.14	0.0003 [(a)]	8.45	8.43	1.98
L	0.30	4.29	25.42	40.08	0.0029 [(b)]	11.52	9.14	2.10
P-Value	*>.10*	*>.10*	*>.10*	*>.10*	*0.10*	*>.10*	*>.10*	*>.10*
SEM [10]	0.26	2.87	15.29	17.84	0.0024	4.17	3.21	2.19

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of digesta 12.27 (S), 12.24 (C), 12.72 (E), 12.18 (L), $P > 0.10$, SEM 0.84; [2] Entero, Enterobacteria; [3] Ccl1, *Clostridium* cluster I species; [4] Lacto, *Lactobacillus* species; [5] Strepto, *Streptococcus* species; [6] Bifido, *Bifidobacterium* species; [7] BaPr, *Bacteroides* and *Prevotella* species; [8] Ccl4, *Clostridium* cluster IV species, [9] Ccl14a, *Clostridium* cluster XIVa species; [10] SEM, standard error of the mean. [(a)-(b)]Values in same column with different superscripts are different (P<0.10).

differences ($P > 0.10$) in the abundance of these bacterial groups were observed. *Lactobacillus* spp. and enterobacteria were found at lower levels around 0.5 to 2% and also not affected by treatment ($P > 0.10$). *Streptococcus* spp. (TRFs 48, 460 and 553) were detected in highest relative abundance ($P < 0.05$) between 30 to 50% of total TRF peak area, and was numerically highest with 15 to 40% of total bacteria in qPCR. Interestingly, these bacteria were found to be in lowest ($P < 0.05$) abundance in digesta for animals in treatment S via TRFLP analysis and numerically lowest in this group as established by qPCR. *Bifidobacterium* was only detected at low levels via qPCR with a trend to being lowest ($P = 0.10$) in treatment E animals and highest in treatment L animals (Table 7.4).

7.4.3. Microbiota in Colonic Mucosa

Similar to findings in the colon content, the mucosal abundance of inoculant species *C. perfringens* and *S. infantarius* (Table 7.5) was not significantly altered by treatment ($P > 0.10$). However, *L. mucosae* was significantly reduced ($P < 0.01$) in treatment L colon mucosa compared to colon mucosa of all other treatment groups. For *C. perfringens* and *S. infantarius*, relative abundance in colon mucosa was about 10 fold lower and 20 fold higher, respectively, compared to respective species abundances in colon digesta, whereas the magnitude of *L. mucosae* abundance was not impacted by radial location. Total bacteria counts in large intestinal mucosa were significantly lower ($P < .001$) with an average of 8.4 $\log_{10}$ copies / g of mucosa (Table 7.6) compared with the large intestine digesta which averaged 12.4 $\log_{10}$ copies / g of content (Table 7.4). *Clostridium* cluster I were found to be highest ($P = 0.05$) in mucosa of animals belonging to treatment E, even though *C. perfringens* counts did not vary between treatments ($P > 0.10$; Table 7.5). Similar to findings for digesta, *Bifidobacterium* spp. as a percent of total bacteria was lowest ($P < 0.01$) in treatment E mucosa (Table 7.6).

Table 7.5. Mean relative abundance (% of total bacteria) of inoculated bacteria determined by qPCR in colon mucosa collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups [1]

Treatment	Cperf [2]	Sinfa [3]	Lmuco [4]
S	0.617	0.001336	0.437 [b]
C	0.784	0.001575	0.356 [b]
E	0.515	0.001240	0.468 [b]
L	0.839	0.001419	0.059 [a]
P-Value	*>.100*	*>.100*	*<.001*
SEM [5]	0.256	0.001812	0.113

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of mucosa 8.36 (S), 8.35 (C), 8.43 (E), 8.39 (L), $P > 0.10$, SEM 0.18; [2] Cperf, *Clostridium perfringens*; [3] Sinfa, *Streptococcus infantarius*; [4] Lmuco, *Lactobacillus mucosae*; [5] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different (P<0.05).

Table 7.6. Mean relative abundance (% of total bacteria) for selected major taxonomic groups in colon mucosa of pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	Entero [2]	Ccl1 [3]	Lacto [4]	Strepto [5]	Bifido [6]	BaPr [7]	Ccl4 [8]	Ccl14a [9]
S	0.83	3.54 [ab]	17.77 [b]	37.17	0.080 [b]	0.139	1.07	0.15
C	2.12	3.27 [a]	12.30 [b]	45.71	0.048 [ab]	0.172	1.44	0.18
E	2.25	7.46 [b]	20.21 [b]	33.64	0.010 [a]	0.191	1.00	0.21
L	1.29	4.80 [ab]	5.96 [a]	41.04	0.018 [ab]	0.223	1.26	0.17
P-Value	*>.10*	*0.05*	*0.001*	*>0.100*	*0.004*	*>.100*	*>.10*	*>.10*
SEM [10]	1.50	1.60	4.07	10.11	0.010	0.147	0.64	0.16

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of mucosa 8.36 (S), 8.35 (C), 8.43 (E), 8.39 (L), $P > 0.10$, SEM 0.18; [2] Entero, Enterobacteria; [3] Ccl1, *Clostridium* cluster I species; [4] Lacto, *Lactobacillus* species; [5] Strepto, *Streptococcus* species; [6] Bifido, *Bifidobacterium* species; [7] BaPr, *Bacteroides* and *Prevotella* species; [8] Ccl4, *Clostridium* cluster IV species, [9] Ccl14a, *Clostridium* cluster XIVa species; [10] SEM, standard error of the mean; [a-b]Values in same column with different superscripts are different (P<0.05).

To determine whether or not variation on a species level within groups was present, additional species specific qPCR analysis was performed (Table 7.7). *Bifidobacterium thermacidophilum* was found at very low levels in mucosa. It was the only bacterial target that was negatively correlated ($P < 0.01$, $R^2 = 0.90$) with animal performance (Chapter 6, Table 6.7). Highest *B. thermacidophilum* levels ($P < 0.01$) were found in mucosa of treatment S animals, lowest levels in treatment E and L animals with treatment C being intermediate.

Mucosa from Treatment L pigs showed the lowest ($P = 0.001$) relative abundance of TRFs corresponding to *Lactobacillus* spp. (Table 7.6). This was in agreement with species based qPCR enumeration of *L. mucosae* (Table 7.3), *L. amylovorus* and *L. johnsonii* (Table 7.7) in colon mucosa of treatment L pigs, which were all lower ($P < 0.05$) in L pigs compared to pigs in other treatments, whereas *L. reuteri* abundance was not affected ($P > 0.10$). Although there was no difference ($P > 0.10$) in *Bacteroides / Prevotella* group analysis (Table 7.6), *B. fragilis* tended to be highest ($P = 0.09$) in treatment C mucosa compared to all other treatments (Table 7.7). Furthermore, treatment E animals showed the highest ($P = 0.06$) relative abundance of *P. buccalis* compared to treatment C and L animals with treatment C being intermediate.

7.4.4. Colon Morphology and Goblet Cell Number

Colonic crypts were found to be significantly ($P = 0.001$) deeper in treatment S animals (Table 7.8). Treatment L and S animals presented with the largest number ($P < 0.05$) of goblet cells per crypt (Table 7.8). Representative cross sections can be found in Figure 7.1. Red or purple stained goblet cells secreting α-glycol-rich neutral mucin were not observed.

Table 7.7. Mean relative abundance (% of total bacteria) of selected bacterial species determined by qPCR in colon mucosa collected at 28 d of age for pigs in the *S. infantaris* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

	Lactobacillus spp.			*Streptococcus* spp.		*Bifidobacterium* spp.		*Bacteroides / Prevotella* spp.	
Treatment	Lamyl [2]	Ljohn [3]	Lreut [4]	Sequi [5]	Ssuis [6]	Banim [7]	Btham [8]	Bfrag [9]	Pbucc [10]
S	0.246 [ab]	0.060 [b]	0.417	0.032 [(a)]	0.054	0.0026 [(b)]	0.00034 [b]	0.026 [(a)]	0.038 [(ab)]
C	0.547 [b]	0.034 [ab]	0.498	0.080 [(b)]	0.082	0.0024 [(b)]	0.00015 [ab]	0.052 [(b)]	0.028 [(a)]
E	0.795 [b]	0.063 [b]	0.445	0.051 [(ab)]	0.044	0.0020 [(ab)]	0.00005 [a]	0.030 [(a)]	0.068 [(b)]
L	0.103 [a]	0.010 [a]	0.445	0.029 [(a)]	0.070	0.0011 [(a)]	0.00001 [a]	0.027 [(a)]	0.024 [(a)]
P-Value	*0.012*	*0.021*	*0.963*	*0.090*	*0.124*	*0.059*	*0.004*	*0.089*	*0.056*
SEM [11]	0.101	0.007	0.100	0.027	0.012	0.0004	0.00012	0.015	0.008

[1] separation of means by Tukey HSD, total bacteria $\log_{10}$ count/g of mucosa 8.36 (S), 8.35 (C), 8.43 (E), 8.39 (L), $P > 0.10$, SEM 0.18; [2] Lamyl, *Lactobacillus amylovorus*; [3] Ljohn, *Lactobacillus johnsonii*; [4] Lreut, *Lactobacillus reuteri*; [5] Sequi, *Streptococcus equinus*; [6] Ssuis, *Streptococcus suis*; [7] Banim, *Bifidobacterium animalis*; [8] Btham, *Bifidobacterium thermacidophilum*; [9] Bfra *Bacteroides fragilis*; [10] Pbucc, *Prevotella buccalis*; [11] SEM, standard error of the mean; [a-b]Values in same column with different superscripts are different (P<0.05); [(a)-(b)]Values in same column with different superscripts are different (P<0.10).

Table 7.8. Mean depth (µm) of colonic crypts and number of Goblets cells per colon for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups. [1]

Treatment	Crypt	
	depth [2]	acidic goblet cells [3]
S	431.2 [b]	67.8 [b]
C	387.3 [a]	52.7 [a]
E	371.2 [a]	49.4 [a]
L	377.3 [a]	68.0 [b]
P-Value	*0.001*	*0.037*
SEM [4]	31.3	3. 9

[1] Individual animal data averaged over 10 observations, separation of means by Tukey HSD; [2] in µm; [3] acidic carboxylated, sialated and/or sulfated mucin containing goblet cells averaged over 10 crypts; [4] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P<0.05$).

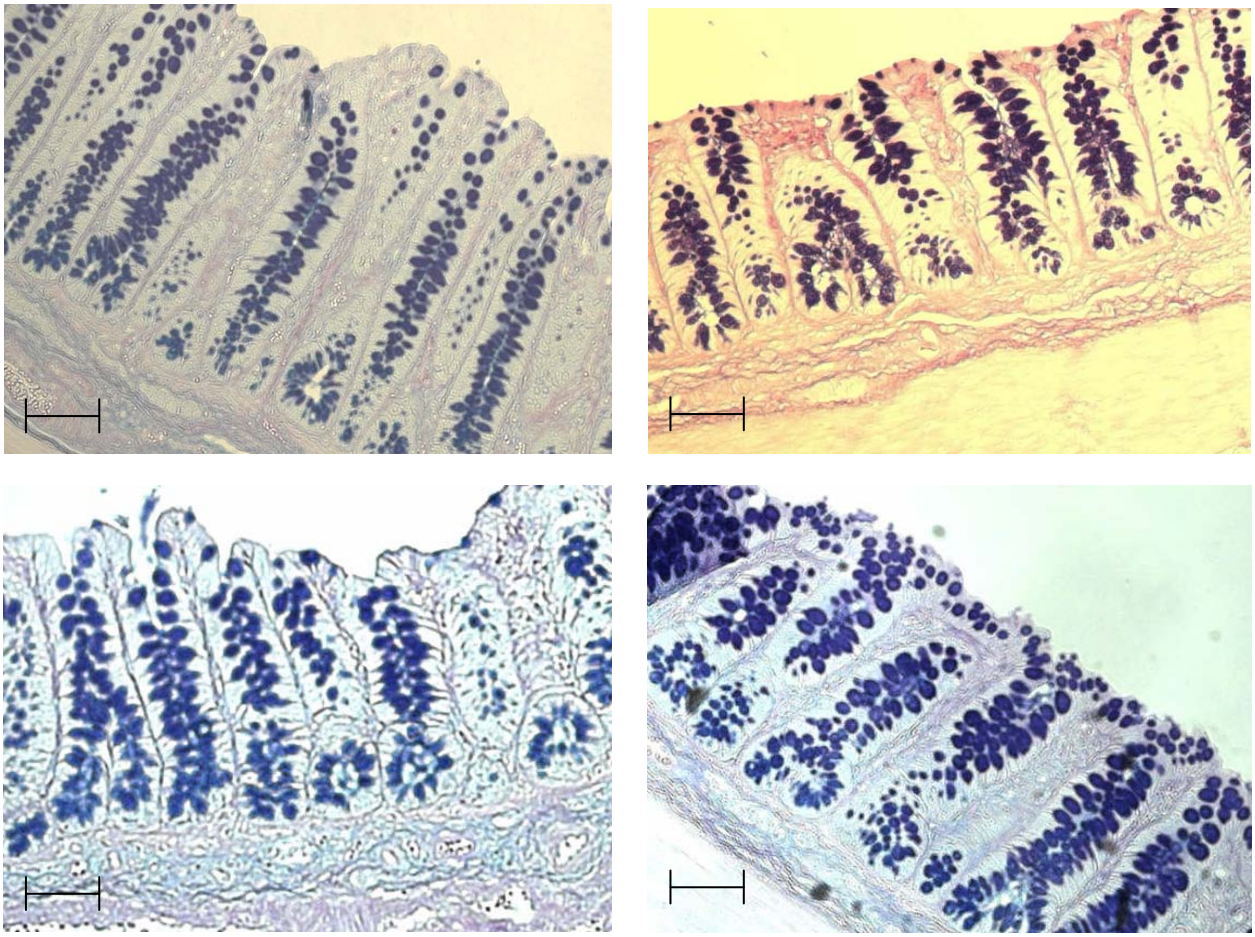

Figure 7.1. Gill's H&E and AB2.5/PAS stained colon sections from treatment S (top left), C (top right), E (bottom left) and L (bottom right), bar indicates 100 µm.

7.4.5. Large Intestine Host Gene Expression

Expression level of selected mucin genes is shown in Table 7.9. A clear pattern of expression of mucin genes in colon was evident for Treatment E pigs. For both, secreted (*Muc2*) and transmembrane (*Muc1*, *Muc4*, *Muc13* and *Muc20*) mucins, gene expression was significantly ($P < 0.05$ for *Muc1*, *Muc4*, *Muc20*) or numerically ($P > 0.10$ for *Muc1*, *Muc13*) lowest in treatment E pigs. Highest mucin gene expression levels were observed for pigs in treatment S and L with the exception of *Muc13* which was numerically ($P > 0.10$) expressed at the highest level in colon of treatment C pigs. Gene expression of secreted and total transmembrane mucus was positively correlated ($P < 0.001$, $R^2 = 0.97$) to goblet cell numbers per crypt.

Similar to mucin gene expression, *TLR2*, *TLR4*, *NFKB1*, *NFKB2* and *NFKBIA* transcript abundance was lowest ($P < 0.05$) in treatment E animals, intermediate in treatment C and L animals and highest in treatment S animals (Table 7.10). Although TLR and NFκB related gene expression were affected, no differences ($P > 0.10$) between treatments were observed for TIRAP/MyD88-mediated expression of pro-inflammatory *IL-1β*, *IL-6*, *IL-8*, *TNFα*, or anti-inflammatory (*IL-10*) cytokines. Similarly, gene expression via the TRAM/TRIF dependent signaling pathway via *IRF3*, *IFNγ* and *FasL* was not affected ($P > 0.10$; data not shown). Glucagon-like peptide-2 receptor (*GLP-2R*) related gene expression was not impacted by treatment ($P > 0.10$; data not shown).

7.5. Discussion

Few studies have examined the impact of variation in early postnatal succession dynamics on the establishment of intestinal microbiota in the post-weaned pig (Mulder *et al* 2009; Schmidt *et al.*, 2011). Of the studies examining the effect of variation in succession patterns, none have focused on microbial and host responses in distal gut. Since neonatal monoassociation had an

Table 7.9. Mean mucus related gene expression in the colon collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

	Transmembrane Mucins					Secreted Mucin
Treatment	*Muc1*	*Muc4*	*Muc13*	*Muc20*	Total [2]	*Muc2*
S	22149 [(b)]	2.177 [b]	157.3	2627 [ab]	24935 [b]	15351 [b]
C	18086 [(ab)]	1.961 [ab]	348.3	2644 [ab]	21080 [ab]	8247 [ab]
E	15814 [(a)]	0.856 [a]	169.8	1919 [a]	17904 [a]	5857 [a]
L	21232 [(b)]	1.204 [ab]	274.2	5131 [b]	26638 [b]	12829 [b]
P-value	*0.071*	*0.009*	*>.100*	*0.011*	*0.043*	*0.001*
SEM [3]	4770	0.801	143.0	1559	8213	2582

[1] Data in gene copies per 1000 *GAPDH*, separation of means by Tukey HSD; [2] Total, sum of attached mucin gene copies per 1000 *GAPDH*; [3] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P<0.05$); [(a)-(b)]Values in same column with different superscripts are different ($P<0.10$).

Table 7.10. Mean bacterial sensing and immune status related gene expression in piglet colonic intestinal tissue collected at 28 d of age for pigs in the *S. infantarius* (S), *C. perfringens* (C), *E. coli* (E) and *L. mucosae* (L) treatment groups.[1]

Treatment	*TLR2* [2]	*TLR4* [3]	*NFKB1* [4]	*NFKB2* [5]	*NFKBIA* [6]
S	94.62 [b]	510.8 [c]	3074 [b]	71.03 [b]	610.8 [b]
C	58.36 [ab]	305.4 [ab]	2407 [ab]	46.86 [ab]	416.4 [ab]
E	41.21 [a]	212.3 [a]	1317 [a]	28.27 [a]	232.1 [a]
L	70.60 [ab]	365.2 [b]	2300 [ab]	50.92 [ab]	349.7 [ab]
P-value	*0.049*	*0.004*	*0.016*	*0.009*	*0.004*
SEM [7]	25.40	120.8	914	19.60	145.7

[1] Data presented in gene copies per 1000 *GAPDH*, separation of means by Tukey HSD; [2] *TLR2*, toll-like receptor 2; [3] *TLR4*, toll-like receptor 4; [4] *NFKB1*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; [5] *NFKB2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; [6] *NFKBIA*, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; [7] SEM, standard error of the mean; [a-b] Values in same column with different superscripts are different ($P<0.05$).

impact on microbiota composition post weaning in the upper gut (Chapter 6), treatment differences in microbiota composition in the hindgut of post weaned pigs is likely. However, the colonic environment and physiological function vary considerably from jejunum. The small intestine is the major nutrient digestion and absorption site and the major site of induction of adaptive immune responses (Brandtzaeg and Pabst, 2004). The main function of the large intestine is to facilitate water absorption and to provide energy in form of volatile fatty acids from microbial fermentation (Heneghan, 1988) with butyrate being the preferred fuel for colonocytes (Dukes and Reece, 2004). The higher number and greater complexity of microbiota in the colon (Inoue *et al*., 2005) makes the hindgut a reservoir for pathogens, including for example *C. difficile, Salmonella* and *Brachyspira* (Straw *et al.*, 2006). Therefore, the colonic epithelium is protected by a thick mucus layer (Atuma *et al.*, 2001), and microbiota sensing via isolated lymphoid follicles induces inflammation only (Brandtzaeg and Pabst, 2004). The increased mucus protein secretion in the hindgut compared to the small intestine comes at a maintenance cost for colonocytes (Dukes and Reece, 2004). Those histological and functional differences result in two different ecological niches for bacterial growth with different but equally important contributions to host metabolism.

The use of a gnotobiotic model, despite significant technical challenges, provided successful control of microbial succession in order to determine subsequent effects on the post-weaning piglet. The choice of inoculant strains for this study was based on a selection of biochemically diverse bacteria identified in piglet intestine between 6 h and 3 d of age (Petri *et al.* 2010). Monoassociation with four distinct bacterial species for the first four days of age resulted in significantly different growth rate of pigs post weaning (Chapter 6). Here we show that performance differences were associated with altered colonic microbial profiles, gut histology

and expression of microbial sensing genes including toll-like receptors, NFκB transcription factors and mucin genes.

7.5.1. Microbial Differences

As observed in jejunal digesta and mucosa taken from the same pigs (Chapter 6), monoassociation with different bacterial species for the first 4 postnatal days significantly affected bacterial colonization patterns in colon digesta and mucosa. Whether the monoassociated species directly influenced its own colonization or the colonization of closely related bacteria (e.g. within same genus) was of particular interest. Several TRFs associated with *Streptococcus* species were in low abundance in digesta from the *S. infantarius* monoassociated group, however, qPCR enumeration of this taxonomic group and several species within the group, including *S. infantarius* did not show treatment differences in colonization patterns. This contrasted our findings in jejunum (Chapter 6) where early postnatal *S. infantarius* monoassociation was correlated with increased relative abundance of *Streptococcus* spp. in postweaning digesta, but not mucosa. For *C. perfringens* inoculated animals, *Clostridium* cluster I bacteria were lowest in postweaning mucosa whereas no other *Clostridium* cluster was affected nor was the relative abundance of *C. perfringens*. This was in contrast to the observation that *Clostridium* cluster I abundance was highest in the jejunal mucosa in *C. perfringens* inoculated pigs (Chapter 6).

The most interesting effect of inoculation treatment was seen in the colonic mucosa for pigs monoassociated with *L. mucosae,* where significantly lower abundance of the taxonomic group corresponding to the monoassociated species was observed. For the *L. mucosae* monoassociated group, a clear pattern of reduced colonization by total *Lactobacillus* spp. including several species with the exception of *L. reuteri* was observed. The consistently lower *Lactobacillus* spp.

at 28 d in the colon mucosa may suggest that early colonization in the colon reduced the tolerance in the colon mucosa for this taxonomic group (Artis, 2008).

Interestingly, *L. reuteri,* which was not reduced in colon mucosa, is the only species of *Lactobacillus* that has been described to induce dendritic cell mediated self-specific host tolerance as demonstrated in *Lactobacillus* free mice (Hoffman *et al.*, 2008; Livingston *et al.*, 2010). Upon initial detection of *L. reuteri* and temporary expression of pro-inflammatory cytokines, *L. reuteri* did not trigger any cytokine expression in spite of high levels in the murine gut after 21 days. This may explain why *L. reuteri* population was unaffected by treatment, although all other *Lactobacillus* species were reduced in abundance. Curiously, however, no similar effect was observed in jejunal mucosa (Chapter 6) suggesting that reduced tolerance is site specific. This might be connected to differences of gut mucosa-associated lymphoid tissue as an induction site for immunological tolerance since *L. mucosae* is the only inoculant species known to attach the mucosal surface of the gut (Roos *et al*., 2000), whereas all other species were not attaching (*S. infantarius* and *C. perfringens* inoculant species) or have been tested to not contain gene markers for mucosal attachment (*E. coli* inoculant).

Microfold or Membranous (M-) cells are responsible for microbe-associated molecular pattern (MAMP) recognition and for B- and T-cell priming in the small and large intestine. In the small intestine, M-cells are mainly found in Peyer's Patches (PPs) as well as in Isolated Lymphoid Follicles (ILFs) whereas in the large intestine, M-cells are part of ILFs only (Brandtzaeg and Pabst, 2004). In direct comparison, it was established that M-cells in Peyer's Patches of the small intestine expressed Human Leucocyte Antigen (HLA)-class II antigens, which are important in tolerance induction to commensal microbiota (Ueki *et al.*, 1995). However, M-cells in ILFs express Intracellular Adhesion Molecule 1 (ICAM-1), which is

believed to play a role in inducing inflammation of colonic mucosa (Ueki *et al.*, 1995). These functional differences in immune induction tissues could potentially be responsible for the differing response in jejunum versus colon to the mucosa attaching *L. mucosae* in our study.

Differences in microbial ecology of the colonic mucosa among treatment groups were also observed that were not directly related to the monoassociated species. *Clostridium* cluster I bacteria were in high abundance in the colonic mucosa of *E. coli* inoculated pigs as observed in jejunal mucosa reported previously. *Bifidobacterium* spp. related counts were detected in low abundances in the colon with treatment effects in colon digesta and mucosa similar to findings in the upper gut (Chapter 6), emphasizing *Bifidobacterium* as a minor bacterial group that was affected by succession differences. Since *Bifidobacterium* was described as an important factor with regard to the hygiene hypothesis (Kirjavainenand and Gibson, 1999; Fanaro *et al.*, 2003; Kelly *et al.*, 2007), this may be a finding if interest.

7.5.2. Host Differences

As reported in Chapter 6, growth rate of pigs between 7 and 28 days of age was lowest for pigs monoassociated with *S. infantarius* and highest for pigs monoassociated with *E. coli* or *L. mucosae*. Similar to our observations in jejunum mean crypt depth in colon was significantly increased in *S. infantarius* monoassociated pigs, correlating with reduced growth rate. Also, similar to findings in the jejunum pro- and anti-inflammatory cytokine expression (*IL-1β*, *IL-6*, *TNFα* vs. *IL-10*) was not affected by treatment. However, a consistent pattern in the profile of expression of genes associated with microbial sensing and inflammation was observed which was most striking for pigs monoassociated with *E. coli* and *S. infantarius.*

Pigs in the *E. coli* group, which showed high growth rate, demonstrated the lowest toll-like receptor (*TLR2*, *TLR4*), NFκB transcription factor complex (*NFKB1*, *NFKB2*, *NFKBIA*) and mucin (*Muc2*, *Muc4*, *Muc20*) gene related expression. Although interpretation based on gene

expression alone can omly be speculative, this expressin profile in *E. coli* could indicate a low sensing and high tolerance of microbiota (Grabig *et al.,* 2005). The profile of expression was similar in the jejunal mucosa for *E. coli* monoassociated pigs as previously reported (Chapter 6). Both jejunal and colonic mucosa demonstrated high abundance of potentially mucus attaching *Clostridium* cluster I bacteria (Arbuckle, 1972) which might be associated with a challenge to the host epithelium since *Clostridium* cluster I often produce toxins (Heikinheimo and Korkeala, 2005) and/or enterotoxic metabolites like H_2S (Bergey and Holt, 1994). In contrast to *E. coli* monoassociation, *S. infantarius* inoculated animals showed the lowest growth rate, had the highest expression of toll-like receptors, NFκB complex genes and Mucin (*Muc1, Muc2, Muc4*) genes. This gene expression profile was similar to that observed in jejunum for *S. infantarius* monoassociation, where *TLR4, NFKB1, MUC2* expression were highest in this treatment. This could suggest that *S. infantarius* inoculated animals had a high level of sensitivity or level of surveillance of the microbial environment compared to *E. coli* inoculated animals, which demonstrated a gene expression profile consistent with low surveillance and sensitivity to gut microbiota. The contrasting profiles were correlated with contrasting growth rates for *E. coli* and *S. infantarius* inoculated animals, respectively.

L. mucosae inoculated animals demonstrated a growth rate similar to the *E. coli*-monoassociated group but with a gene expression profile similar to the slower growing *S. infantarius* group. *L. mucosae*-monoassociated pigs showed elevated expression of mucin genes and intermediate expression of toll-like receptors and the NFκB complex genes. This contrast in mucosal gene expression profiles between *E. coli* and *L. mucosae* monoassociated pigs was also observed in jejunum (Chapter 6). Thus a gene expression profile associated with a heightened microbial sensing and mucin production response was associated with both high (*L. mucosae*)

and low (*S. infantarius*) growth. Furthermore, low microbial sensing and mucin expression was also associated with high (*E. coli*) performance.

Although no explanation for this variation in response profiles is evident, it is possible that elevated butyrate content in colon digesta of *L. mucosae* monoassociated pigs may have played a role. Butyrate in the hindgut is mainly derived by fiber degradation of *Clostridium* cluster IV and XIVa (Barcinella *et al.*, 2000; Pryde *et al.*, 2002; Tsukahara *et al.*, 2002) with higher counts in the hindgut compared to the small intestine and stomach (Chapter 4, Castilo *et al.*, 2006a). Aside of direct fueling of the colonocytes by butyrate (Heneghan, 1988), butyrate is anti-inflammatory (Segain *et al.*, 2000), impacts host cell proliferation via histone deacetylation (Boffa *et al.* 1978) and *Muc2* gene expression of goblet cells in the absence of glucose (Gaudier *et al.*, 2008). These effects of butyrate, which were highest in colon digesta of *L. mucosae* inoculated animals in the current study, may have contributed to improved growth rate and mucosal barrier health of treatment L animals, highlighting the importance of the hindgut for animal health and performance. On the other hand, the gene expression profile was comparable in colon and small intestine of *L. mucosae* inoculated animals and given the very low levels of butyrate a direct role of this organic acid on small intestinal epithelium is unlikely.

7.6. Conclusions

Pigs inoculated with *S. infantarius* during the early postnatal period presented a postweaning mucosal gene expression profile that could be interpreted as representing high surveillance and sensitivity to the microbial environment. In contrast, *E. coli* inoculated animals show consistent down regulation of genes related to microbial detection and mucus expression, as well as improved growth rate compared to *S. infantarius* inoculated animals. This growth rate improvement might be derived by a lower energy need of *E. coli* inoculated animals for immune

related function (Martin II *et al.*, 2003) and possibly improved appetite (Liu *et al*, 2008). However, pigs monoassociated with *L. mucosae* in the early postnatal period showed similar growth rates to *E. coli* inoculated animals, but with an gene expression profile more similar to *S. infantarius*. The performance difference between *L. mucosae* and *S. infantarius* inoculated animals may be explained by the elevated butyrate levels in colon of *L. mucosae* monoassociated pigs, affecting colonocytes metabolism and modulating inflammatory response. In any case, there was general agreement in the relative expression of toll-like receptors, NFκB complex genes and mucin genes between jejunal and colonic mucosa. Results indicate that early postnatal management to control the gastrointestinal microbial succession profile could have implications for gut health and performance of animals post weaning.

8.0 GENERAL DISCUSSION AND CONCLUSION

Building on findings determined via culture-based (Ducluzeau 1985; Fuller *et al.* 1978; Pederson and Tannock, 1989; Smith, 1965, Swords *et al.* 1993) and molecular-based analysis methods reported in previous studies (Inoue *et al.* 2005, Konstantinov *et al.* 2006; Mikkelsen *et al.* 2003), a more detailed and complete succession pattern for neonatal to preweaned pigs was established. It was determined that aside of the previously documented initial predominance of Enterobacteria and *Clostridium* cluster I in the digestive tract, and the later onset of a predominance of *Lactobacillus*, there was an intermediate phase of *Streptococcus* predominance in the gastro-intestine tract of day old pigs (Chapter 3). Additionally, specific details regarding digestive tract microbiomes were revealed. Lowest bacterial counts were determined in duodenum not stomach of early and late preweaned pigs, differing from findings of others (Castillo *et al.* 2006a, 2007) for stomach. The difference here were most likely a bias introduced by immediate snap freezing of samples, not allowing for DNA from dead microorganisms to be degraded in the stomach contents due to lower pH and low DNA degrading enzyme secretion by host and commensal microbiota in contrast to the intestine (Duke and Reece, 2004). Microbial community differences could be determined around two days of age (Chapter 3), which coincides with the change from colostrum to mature sow milk (Fan 2003), indicative of an maternal impact on commensal microbial via food supply in suckling pigs. Regarding specific bacterial differences, *L. delbrueckii* was determined in upper digestive tract only. *Bacteroides* and *Prevotella* were predominantly detected in the hindgut, and elevated counts of *Bifidobacterium* and *Clostridium* cluster XIVa species were detected in jejunal mucosa (Chapters

3 and 4), which was similar to findings in humans (Pérez *et al.*, 1997; Wang *et al.* 2003). *Clostridium* cluster I, which was determined to mostly consist of *C. perfringens*, showed a decline over time in the digestive tract. Interactions between locations and intra-group variations were observed for *Streptococcus* and *Lactobacillus* groups (Chapter 4), the latter were also observed by Konstantinov *et al.* (2006), stating a growing predominance of *L. amylovorus* (they described as *L. sobrius*, revised by Jakava-Viljanen *et al.* 2008) and a reduced relative abundance of *L. reuteri* within the *Lactobacillus* genus comparing neonatal and late pre-weaned pigs.

A comparison of digesta and mucosal microbial profile identified radial differences in microbial colonization. For jejunum, total eubacterial 16S rRNA gene copies were markedly higher per gram digesta versus per gram mucosal scraping, which was in agreement Castillo *et al.* (2006a). Furthermore, the reduction of total bacterial counts towards the host epithelium was more pronounced in the colon compared to the jejunum (Chapter 4). This is most likely due to the fact that host microbiota detection in the hindgut exclusively triggers inflammation, whereas tolerance can be induced via Peyer's Patches in the small intestine (Brandtzaeg and Pabst, 2004). This is mediated via host defensin secretion, e.g. IgA (Brandtzaeg and Pabst, 2004), and due to greater host mucus secretion in the colon compared to the jejunum (Atuma *et al.* 2001, Chapter 6 vs. Chapter 7). In addition, the mucosal microbiota community was not just a mirror of the digesta microbiota in lower abundances (Chapter 4). Significant differences were determined, for example, due to the drastic decrease of *Enterococcus* per gram of colon mucosa versus colon digesta, whereas *Bacteroides/Prevotella* counts were not affected by radial location in both, jejunum and colon (Chapter 4). In hindsight, a more controlled setup and the same age piglets collected from each sow might have been an improvement to the study design. In addition, it would be an improvement to have sows of the same parity with synchronized farrowing of all

animals in the same room. A more controlled and clean research environment could have been of benefit to animal management, but could have in turn increased inter-animal variability similar to observations of Thompson *et al.* (2008). Furthermore, measurement of physical growth parameters of piglets might have provided additional insights.

After all, 454-pyrosequencing of the gut microbiome might have revealed a more complete picture of microbial succession and between longitudinal and radial gut locations (Chapters 3 and 4) compared to the sequence library and qPCR approach. However, sequence libraries were chosen to identify microbiota to the species level in order to select inoculant species for microbial programming, and qPCR to be able to quantitatively analyze microbial data. High sampling frequency as early as 6 hours post-partum was chosen to identify and isolate key players in neonatal microbial succession. It was also attempted to obtain an insight into the microbiota of the sow birth canal as the earliest exposure to neonatal piglets as suggested in pigs by Ducluzeau (1985) and supported by findings of Hill *et al.* (2005b) in humans. However, no bacterial DNA could be amplified from vaginal swabs taken from sows pre, peri and post farrowing (Chapter 5) which was similar to findings of Bara *et al.* (1993). On the other hand, Microbial analysis of sow fecal matter revealed similarities to microbes determined in piglets (Chapters 3 and 5), emphasizing the environmental impact on microbial succession in neonatal animals.

In order to further study the impact of the isolated early predominant species in an animal study with limited exposure in neonatal pigs, as suggested to be key to immune development in later life (Kelly *et al.* 2007, Mulder *et al.* 2009), two experimental models were evaluated as approaches to study the effect of differences in early postnatal microbial succession profile on postweaning microbial ecology and gastrointestinal gene expression. A gnotobiotic approach

was necessary to successfully demonstrate post-weaning differences due to differences in neonatal ecology and to minimize environmental effects. The snatch farrow approach (chapter 5) was attempted as a low cost and simplified protocol alternative germ-free derivation of piglets by caesarean section. However, the unpredictability of exact farrowing times, litter size, period between the first and last pig of each litter and complications in the farrowing process itself due to the high irritation level of the sows, as well as differences in transfer times to the pre-prepared isolators leading to strong litter effects (Chapter 5) that were otherwise not observed (Chapter 3, 4, 7, 8).

Although the trial design in Chapter 5 supported the hygiene hypothesis as proclaimed by Kelly *et al.* (2007), it showed also high variability between litters of piglets. In addition, the non-inoculated treatment group, showed high similarly in gut microbiota to co-housed animals similar to Thompson *et al.* (2008). Despite the successful establishment of the model, the snatch farrow approach was dismissed due to a lack of differences in intestinal microbial profiles post-weaning combined with the difficulty in predicted timing and duration of natural farrowing.

As an alternative, the gnotobiotic approach was chosen as described by Shirkey *et al.* (2006) and Willing (2007) using modified methods. Sterile piglets were derived via cesarean section with immediate transfer of piglets through an iodine bath into a sterile transfer unit, and from there into treatment specific sterilized isolators. The initial attempt succeeded in regard to deriving gnotobiotic piglets. However, the difficulty of collecting sufficient amounts of sow colostrum resulted in insufficient passive immunization of piglets leading to drastic mortalities within 10-15 days after birth without presence of any overt pathogens. To prevent further causalities, the trial was aborted. For the second gnotobiotic study, a large amount of colostrum was collected and shipped for irradiation. In addition, sow colostrum was intended to be

supplemented with sterile porcine plasma and freeze dried bovine colostrum, as well as with medium-chain triglycerides (MCT) in order to boost passive immunization and to prevent immunoglobulin utilization for maintenance energy of the host (Dukes and Reece, 2004). Unfortunately, probably associated with a communication error, the colostrum spoiled while awaiting irradiation at a distant facility. Out of immediate need (impending planned caesarian section surgeries), a porcine colostrum replacer solely based on commercial bovine colostrum, porcine plasma and MCT was developed, leading to the improvements in passive immunization without use of porcine colostrum. A larger scale trial with four treatments and six piglets per treatment was accomplished. The most prevalent microorganisms at first predominance in microbial succession (Chapter 3) were selected, cultivated and used in monoassociated gnotobiotic pig model. These four organisms were *E. coli*, *C. perfringens, S. infantarius* and *L. mucosae,* also reflecting a wide array of distinguishing attributes: non-pathogenic non-attaching *E. coli* was the only predominant Gram negative organism (Casey and Bosworth, 2009), *C. perfringens* type A as commensal, non-epithelial surface adhering, α-toxin producing organism (Heikinheimo and Korkeala, 2005), *S. infantarius* is a non-hemolytic, non-mucosal surface adhering Gram positive organism (Bergey and Holt, 1994) and intestinal epithelium adherent, non-toxin producing *L. mucosae* (Roos *et al.*, 2000). Animals were kept until after weaning, and differences due to neonatal inoculation and monoassociation determined in differing microbiomes within the pig. Differences in gastro-intestine digesta and mucosa commensal bacteria composition relative to treatment are summarized in Table 8.1, and variation in host histology, digestive capacity, mucus expression and microbiota detection are summarized in Table 8.2. Improved animal growth

Table 8.1. Overview of effect of monoassociation with *S. infantarius, C. perfringens, E. coli* or *L. mucosae* from 0 to 4 days of age in pigs, on relative abundance of selected bacteria as determined by qPCR, in digesta and mucosa of jejunum and colon at 28 days of age after weaning to a cereal-based diet. [1]

Bacterial target	Location	*Monoassociation Treatment*							
		S. infantarius		*C. perfringens*		*E. coli*		*L. mucosae*	
		digesta	mucosa	digesta	mucosa	digesta	mucosa	digesta	mucosa
Streptococcus infantarius	jejunum	x	x	x	x	x	x	x	x
	colon	x	x	x	x	x	x	x	x
Streptococcus	jejunum	H	H	L	L	M	M	M	M
	colon [2]	L	x	H	x	H	x	H	x
Clostridium perfringens	jejunum	x	x	x	x	x	x	x	x
	colon	x	x	x	x	x	x	x	x
Clostridium cluster I	jejunum	(M)	M	(L)	H	(M)	H	(H)	L
	colon	x	M	x	L	x	H	x	M
Lactobacillus mucosae	jejunum	x	x	x	x	x	x	x	x
	colon	x	H	x	H	x	H	x	L
Lactobacillus	jejunum [2]	L	x	L	x	L	x	H	x
	colon	x	H	x	H	x	H	x	L
Enterobacteria	jejunum	H	M	H	L	M	L	L	H
	colon	x	x	x	x	x	x	x	x

[1] H, high, M, medium, L, low; ( ), values in brackets show statistical trend with $0.10 \geq P > 0.05$; x, no difference with $P > 0.10$;
[2] jejunum and colon digesta TRFLP data comparison shown here (Table 6.12, Table 7.4).

Table 8.2. Overview of effect of monoassociation with *S. infantarius, C. perfringens, E. coli* or *L. mucosae* from 0 to 4 days of age in pigs, on relative immune related gene expression level in mucosa of jejunum and colon at 28 days of age after weaning to a cereal-based diet. [1]

Gene target	Location	*S. infantarius*	*C. perfringens*	*E. coli*	*L. mucosae*
TLR2	jejunum	L	L	L	H
	colon	H	M	L	M
TLR4	jejunum	H	M	L	H
	colon	H	M	L	M
NFKB1	jejunum	(H)	(M)	(L)	(M)
	colon	H	M	L	M
NFKB2	jejunum	M	L	L	H
	colon	H	M	L	M
NFKBIA	jejunum	L	L	L	H
	colon	H	M	L	M
Muc1	jejunum	-	-	-	-
	colon	(H)	(M)	(L)	(H)
Muc2	jejunum	H	M	L	M
	colon	H	M	L	H
Muc4	jejunum	-	-	-	-
	colon	H	M	L	H
Muc13	jejunum	(L)	(M)	(M)	(H)
	colon	x	x	x	x
Muc20	jejunum	M	M	H	L
	colon	M	M	L	H

[1] H, high, M, medium, L, low; ( ), values in brackets show statistical trend with $0.10 \geq P > 0.05$; x, no difference with $P > 0.10$.

performance was observed due to inoculation of pigs with *E. coli* and *L. mucosae*, the latter being similarly established by Davis *et al.* (2007) due to *L. brevis* supplement feeding to nursery pigs, with most mature intestine histology and mucus composition. Similar to findings of Siggers *et al.* (2008), the administration of *Lactobacillus* reduced enterobacteria and *Clostridium*. Also to similar to the findings in the current experiment was the up-regulation of nutrition and mucin related gene expression (Siggers et al. 2008). Expression of neutral mucins and highest butyrate concentrations in large intestine digesta were further indicators for a more mature intestine due to *L. mucosae* inoculation as described by others (Castillo *et al.* 2006a; Kien *et al.* 2006 and 2008; Lancorn *et al.* 2010). In contrast, *S. infantarius* inoculation resulted in lowest growth performance, the least effective nutrition related gene expression in the small intestine, the least mature intestine histology, as well as the lowest butyrate concentration in the large intestine. Monoassociation with a Gram positive toxin producing commensal bacterium did improve the situation. *Clostridium perfringens* treated animals performed over all intermediate regarding growth performance, immune development and mucin gene expression but was otherwise unremarkable. Treatment with non-pathogenic and non-adhering Gram negative *E. coli* resulted in lowest MAMP recognition and subsequent NFκB and mucin related gene expression without impact on inflammatory cytokines. This might be due to myeloid dendritic cell detection of commensal bacteria following a similar TLR dependent pathway as in enterocytes. However, it mediates anti-inflammatory *IL-10* expression (Saraiva and O'Garra, 2010), which in turn inhibits *TNFα* (Schwerbrock *et al.*, 2006) creating a greater tolerance of commensal microbiota by muting the NFκB related pro-inflammatory response in enterocytes (Murray, 2005; Kawai and Akira, 2007). To prove this hypothesis might be very difficult due to the complexity of immune related signaling in the host (Kawai and Akira, 2007). However, due to the use of a gnotobiotic

treatment model in the current study, it was possible to determine alterations to the host immune response in general. Findings were mostly similar to experiments conducted by Shirkey *et al.* (2006) and Willing and Van Kessel (2007) where *E. coli* monoassociation did not result in differences in interleukin expression and small intestine crypt histology compared to *Lactobacillus* monoassociation. However, *TLR4* up-regulation led to increased *TNFα* expression (Shirkey *et al.,* 2006; Willing and Van Kessel, 2007). Comparing gnotobiotically reared pigs to monoassociated neonatal pigs and then conventionalized pigs (Chapters 6 and 7), it seemed that neonatal monoassociation with non-toxin or adhesin producing *E. coli* for four days post-partum resulted in desensitization to commensal bacteria. Small and large intestine *TLR* expression was lowest, as was subsequent signaling related gene expression. Since nutrient absorption related gene expression in the small intestine was not as developed as for *L. mucosae* treated animals, animal growth performance seems in part derived by energy preservation by reduced detection of commensal bacteria. However, it is questionable if muting the immune system alone is the solution to improved long-term health, a follow up with a challenge study would be of interest. In said challenge study, it could be determined whether the immune system of *E. coli* monoassociated animals is muted to dysfunctionality, as indicated by the increase of potential toxin and H_2S producing *Clostridium* cluster I spp. in close proximity of the epithelium of treatment E animals (Table 8.1), or if the immune system is still capable to detect and appropriately respond to invasive pathogenic microbiota. It would be of further interest to compare monoassociated with diassociated animals, testing *L. mucosae* and *E. coli* inoculant strains alone and in combination compared with *S. infantarius* inoculated animals, and potentially sow reared animals from the same litter. Furthermore, an extension of trial duration to truly determine long-term effects would be a great achievement. In review of the trial and in

addition to the technical improvements of microbial detection methods via 454-sequencing as stated above, a great improvement to the host specific gene expression methods would be the ability to distinguish *TLR* gene expression between enterocytes versus dendritic cell and macrophage populations. Due to the fact that the same signaling molecules from TLRs to NFκB are resulting in Pro and anti-inflammatory effector molecules, respectively (Kawai and Akira, 2007), this would have allowed for a better insight regarding MAMP recognition resulting in tolerance (Saraiva and O'Garra, 2010) or in immune response (Brandtzaeg and Pabst, 2004). Also, determination of MAPK1 gene expression levels, which are directly connected to trigger inflammation via NFκB mediated signaling (Kawai and Akira, 2007). This might be a key mediator missing in the investigated spectrum of analysis. In addition, the ability to localize TLRs and distinguish between apical and basolateral surface TLRs might have further reduced potential bias. However, at this point in time, these methods are not available.

To summarize the work completed, the objective to establish a comprehensive microbial succession pattern in the conventional and healthy neonatal suckling pig using molecular methods was accomplished (Chapter 3). However, the microbial succession pattern was established for stomach, jejunum and colon only. Four species from predominant bacterial groups within the neonatal bacterial succession were determined, isolated and cultivated. Regarding the objective to gather more detailed information (Chapter 4), findings indicative of a general shift in gut microbiota structure around day two post weaning, were used to determine a complete overview over all longitudinal gut locations between stomach and colon on days 2 and 20 of age. Furthermore, species specific analysis was performed comparing first predominance profiles to day 20 microbiota within *Lactobacillus* and *Streptococcus* spp., revealing shifts within investigated bacterial groups between neonatal and pre-weaned animals. For day 20 of age, a

radial gut comparison was accomplished, comparing jejunum and colon digesta microbiota profiles to their respective mucosa microbiota. The objective to establish a trial design to determine succession differences post weaning was only partially successful. Although shifts in gut microbiota abundance could be determined via the newly developed snatch-farrow approach, results were too inconsistent to use the model for a controlled gnotobiotic succession study. Therefore, a previously established cesarean section based method (Shirkey *et al*. 2006, Willing, 2007) was modified and utilized with success in the second attempt to accomplish the next objective, which was to demonstrate that altered early succession in neonatal pigs using a gnotobiotic model, has effects on microbiota composition, gut histology and host gene expression in the proximal and distal gut of the post weaned pig, respectively. Therefore it was possible to accomplish the overall objective to determine that alterations in the early postnatal microbial succession pattern in intestine after birth up to 4 days of age has long term implications for intestinal microbial ecology and host response as demonstrated in post weaning pigs. It could not be determined if these effects carry through to adulthood as suggested by Kelly *et al*. (2007) but there are indications that the host differences will prevail. It was further indicated that intestinal epithelial cell adherent Gram positive as well as non-adhering Gram negative bacteria play a crucial role in gastro-intestinal development in pigs and most likely in other mammals as well. The intestinal epithelial surface adhering Gram positive organisms seemed to induce histological maturity and stimulated nutrition related gene expression, the non-adhesive Gram negative organisms induced tolerance of commensal bacteria.

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